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Multiple organ dysfunction syndrome (MODS) is most frequent area of late death in trauma patients and a particular problem for combat casualties where conflict conditions may not allow early evacuation to ICU units of extensive treatment. Consequently, delineating mechanisms for ameliorating post-trauma immunosuppression and overproduction of inflammatory cytokines is a major priority. Only the trauma patient subset with both MØ and T cell dysfunctions develop MODS. This patient subsets' MØ are producing large quantities of deregulated and aberrant TNF_α, indicated by increased TNF_α mRNA stability, failure to shed neutralizing TNF, insensitivity to PGE_2 and TGF_β downregulation, as well as predominant production of cell-associated or $mTNF_\alpha$. These aberrant post-injury MØ's ability to activate T cells is also decreased by loss of their IL-12 production and both helper 1 and helper 2 T lymphocyte responses are concomitantly depressed. Dysfunctional T cells fail to appropriately activate or regulate inflammatory MØ allowing exaggerated inflammatory monokines to cause MODS. Aberrant MØ function is detectable as an increase of MØ TNFR (failure to shed the TNFR) and surface expression of $mTNF_\alpha$. Aberrant T lymphocyte activity is also rapidly indicated by depressed CD28 and CD3 expression and concomitant upregulation of CD11b expression. Rapid flow cytometric identification of altered MØ and T cell surface receptor/ligand combinations might serve as an easily implementable technique for screening combat casualties. This contract's data have implicated a combination of T lymphocyte and MØ dysfunctions as responsible for the development of MODS. Both a possible means of rapidly identifying combat casualties at risk of MODS and suggestions of future interventive therapy have been developed as a result of this contract support.

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INTRODUCTION:

Overwhelming infection and multiple organ dysfunction syndrome (MODS) are the most frequent causes of late death in trauma patients. Even patients who survive MODS experience prolonged hospital stays and protracted intensive care needs which would prevent early return to duty for combat casualties. MODS appears to result from excessive proinflammatory monokine production, often secondary to bacterial challenge and frequently associated with suppressed post-trauma immune function. Since the development of MODS is often precipitated by post-injury infection, MODS poses a particular problem for combat casualties where conflict conditions may not allow early evacuation to ICU units or extensive treatment for prevention of bacterial contamination during the early post-injury period. Consequently, delineating mechanisms for ameliorating the post-trauma immunosuppression and overproduction of inflammatory cytokines is a major priority in combat casualty care. Although altered post-trauma T lymphocyte activity and T lymphokine production have been repeatedly linked to poor clinical outcome, it is unclear which post-trauma alterations in T cell function are potentially pathologic or how T cell alterations are linked to the inflammatory cytokine excesses that are the proximal cause of MODS. The research supported by this contract focused on four goals. First, defining the mechanisms and alterations that lead to excessive production of inflammatory cytokines. Second, characterizing those T cell dysfunctions which are pivotal in post-trauma immune depression. Third, delineating what detectable cellular alterations in receptor/ligand expression and/or activation may be responsible for these post-injury MØ and T cell aberrations. Fourth, defining the links between T cell immunosuppression, monokine overactivation, and MODS.

Our hypothesis was developed as a result of experimental results supported by this contract. We hypothesize that altered receptor/ligand triggering in both MØ and T lymphocytes leads to excessive monokine production by MØ and immunosuppression in T lymphocytes and that both these dysfunctions are required for progression to MODS. We postulate that monocyte activation by trauma generated mediators, such as complement split products, fibrin degradation products, substance P (from damaged nerve endings), and coagulation factors, represent an incomplete or aberrant signal which leads to activation of proinflammatory monokines, downregulation of monocyte antigen presenting capacity, and failure to appropriately activate MØ cytokine regulatory genes. This inappropriate partial MØ activation

results in exaggerated proinflammatory monokine production and increases the risk of MODS. However, loss of MØ endogenous inflammatory monokine regulation is not in and of itself enough to produce full progression to MODS. The failure of MØ antigen presenting capacity leads to T cell anergy and this T cell dysfunction contributes to post-injury MODS in two important and necessary ways. First, reduced T cell mediated immunity allows post-injury increases in bacterial and/or viral challenges which further stimulate elevated production of inflammatory cytokines. Second, loss of anti-inflammatory T lymphokine production results in failure of exogenous downregulation of post-trauma mediator induced proinflammatory monokine production, allowing their excessive production. Both aberrant monocyte activation and loss of endogenous regulation, as well as loss of T cell activation and exogenous cytokine regulation, combine to produce deregulated proinflammatory cytokine production which is the proximal cause of MODS. Consequently, any modulating therapy to reduce or prevent MODS in combat casualties must be directed not only to the post-trauma inflammatory monokines, but must also restore T lymphocyte function and regulation of monokines.

METHODS:

Patient population:

Patients with mechanical trauma (injury severity score >25) or thermal trauma (>20% total body surface 2-3° burns) admitted to the University of Massachusetts Medical Center Trauma Unit or Burn Unit, Worcester, were included in the study. Normal controls were tested along with each patient. Volunteers from laboratory and hospital staff at the University of Massachusetts Medical Center served as a normal control panel. The responses of this panel of controls has been standardized over >5 assays during a two year period. Informed consent was obtained from all patients and controls and the study was approved by the Institutional Review Board.

Separation of MØ and T cells:

Peripheral blood mononuclear cells (PBMC) are separated from normals' and patients' blood as described (1). MØ are isolated from PBMC by selective adherence to microexudate-treated plastic surfaces, then T cells are isolated by rosetting with neuraminidase-treated sheep red blood cells (SRBC) (2).

MØ stimulation and assessment of mTNFα/TNFR ratio:

MØ from normals and patients are stimulated with 20μg/ml of muramyl dipeptide (MDP) for 16-18 hr. Supernatants are harvested for the assessment of TNFR by ELISA while the cells are used for the assessment of $mTNF_{\alpha}$ by LM bioassay (3). Increased $mTNF_{\alpha}$/TNFR ratio, which itself has significant correlation to MODS scores, is correlated to any concomitantly identified T cell defect.

T cell stimulation:

T cells are cultured ($2x10^5$ cells/200μl/well) in the presence of PHA (1μg/ml) or anti-CD3 (1μg/50μl/well) + anti-CD4 (1μg/50μl/well) (αCD3/αCD4). Anti-CD3 and anti-CD4 are immobilized as described (2). Lymphokine production is assessed in the culture supernates harvested at 24 hr while proliferation is assessed by [^{3}H] TdR-incorporation at 72 hr of culture. In selected experiments, T cells are stimulated with various other stimuli, such as PMA (10-50ng/ml) + ionomycin (1μM), αCD3 + αCD28, or PMA + ionomycin + αCD28. Since anti-CD3 stimulation inhibits T cell IL-13 generation, PHA (1μg/ml) + PMA (10ng/ml) are used as the stimulus for IL-13 production.

Assessment of cytokines:

Secreted T cell lymphokines (IL-2, IL-4, IL-10, IL-13, and IFN_{γ}) are assessed by commercially available ELISA. T cell and MØ cytokine mRNA levels are assayed in the Mimic PCR system, as detailed in our TNF_{α} paper (4).

Determination of MØ cytokine levels:

$mTNF_{\alpha}$ in the sonicated MØ lysate is assessed in an LM bioassay, while secreted TNF_{α} is assayed both by LM bioassay and ELISA (3). Other cytokines in MØ, such as IL-8, IL-12, TGF_{β}, and IL-15, are assayed by ELISA.

Analysis of TNF-receptors on MØ:

Freshly isolated patients' and paired normals' nonadherent MØ, as well as overnight cultured MØ, both unstimulated and MDP or MDP + IFN_{γ} stimulated, were analyzed for TNF receptor expression as described (5). Other MØ surface receptors, such as $Fc_{\gamma}RI$ (CD64), were assessed using flow cytometry and specific monoclonal antibodies (mAb) as described below.

Analysis of MØ and T cell surface receptor/ligand expression:

T cell expression of different surface molecules, such as CD14, CD11b, CD3, CD4, CD8, CD25, CD28, and TNFR, were analyzed by flow cytometric analysis. Freshly isolated purified T cells from

patients and normals are immediately stained or stained after being kept overnight at 4°C. To determine antigen receptor expression of stimulated cells, the cells are cultured overnight at 10^6 cells/ml media with 2μg/ml PHA or other inducers, as detailed in proposed experiments. Conjugated mAb are obtained from commercial vendors. 5 x 10^4 - 1 x 10^5 are resuspended in 100μl PBS and 5μl conjugated mAb (the equivalent of "one test") is added. For each Ab isotype, an isotypic control is run. Use of mAb of the IgG 1 isotype is preferable, since other isotypes have a higher degree of nonspecific binding (i.e. IgG-2a). Samples are incubated at 4°C in the dark for 30 min and washed twice before flow cytometric analysis. Appropriate gating for positive staining is based on the isotypic control (such that <5% of isotype stained cells are "positive").

Detection of apoptosis:

10^6 MØ depleted PBMC purified T cells or isolated MØ from normals and patients are cultured in 24 well plates in a total volume of 1ml media with 5% FBS. Cells are cultured either unstimulated or stimulated with PHA (2μg/ml), αCD95 (100ng/ml), or PMA(10-50). After 18-21hr culture, the cells are washed and then subjected to the following staining procedure: Propidium Iodide (6): The cells are fixed in 70% cold EtOH for 2-5 min. After washing, they are resuspended in 200μl staining solution (50μg/ml PI, 0.05% Triton X-100 in PBS, 9U/ml DNAse free RNAse) and incubated in the dark at 37°C for 30-45 min. The samples are then read on the flow cytometer. The unstimulated normal control defined the sub-diploid gate and served as the negative control (<5% of the cells in the subdiploid region). Jurkat cells stimulated with PHA (2μg/ ml/10^5 cells) or with anti-Fas (100ng/ml/10^5 cells) serve as positive controls. The amount of apoptosis in stimulated Jurkat cells can be variable but is usually in the range of 20-50%.

Statistical Analysis:

Since many of the immune parameters that we measured here do not follow a normal distribution, we performed our statistical calculations using non-parametric statistics. Although normal volunteers are run simultaneously with every assay presented, the data is not paired in statistical analysis. Therefore a Mann-Whitney non-parametric U test is performed to establish differences among groups for those continuous variables. Chi-square analysis is performed to compare groups with non-continuous or categorical variables. The cutoff and definition of these categorical values is established in advance based on our preliminary data. Specifically, the mTNF/TNFR ratio is considered abnormal if it is >100. The

lymphocyte proliferation assays are considered depressed if there is a proliferation rate lower than 50% of the median found in the normal control panel. Linear regression analysis is used to establish relationships between two variables and logarithmic conversions may be required due to the non parametric distribution of the data. A multiple logistic regression analysis is utilized once there has been a sufficient recruitment of patients to establish a statistical model for prediction of outcome based on these sophisticated immune parameters. The endpoints for this predictive model is not only mortality but also infectious complications and severity of organ dysfunction, utilizing the parameters described by Marshall based on his own population (7).

RESULTS:

During the period 10/1/94 to 5/30/97, 68 patients were monitored, for a total of 285 post-injury time point samples. There were 28 thermally injured patients (>30% 3° burn) and 40 trauma patients (ISS >15) in this group. Altered mitogen responses in the patients' PBMC, which had previously been the characteristics utilized to identify immunosuppression, have been supplanted by assessment of a direct T cell dysfunction, indicated by a failure of purified T cells to respond to a direct stimulus by immobilized anti-CD3 and CD4 (Table I) (2). As initially suggested in the Midterm Report and subsequently published, the trauma patient population appears to be distinguishable on the basis of a development of an intrinsic T cell defect rather than a defect in the response of PBMC (2, 8). Our laboratory has focused on dysfunction in trauma patients' immune responses in an attempt to determine the connection between alterations in post-trauma responses and clinical outcome. We initially focused on changes in the first 24-36 hr post-injury but found that almost 90% of the trauma patients who meet study entry criteria (ISS >15) had a transient initial (day 1-2 post-injury) depression in their overall peripheral blood mononuclear cell (PBMC) responses to mitogen stimulation but that 70-80% of these patients had no adverse clinical outcome (8). We and others demonstrated that patients who had PBMC mitogen depression reoccurring at >5 days post-injury had a much higher incidence of morbidity/mortality due to organ failure and sepsis (≈50%) sometimes reaching a statistically significant correlation (9, 10). However, these depressed post-trauma mitogen responses, although reflecting T cell failure to proliferate, did not always accurately predict or reflect negative clinical outcome. We and others showed that development of negative regulatory activity by patients' isolated T lymphocytes correlated dramatically to poor patient outcome (9, 10).

However, only a small number of patients who had depressed PBMC responses developed negative regulatory T cells and these "suppressor" cells appeared almost concomitant to MODS, making it unclear what, if any, causal relationship was operating. These data caused us to focus, in the first half of the contract, on excessive inflammatory monokine production as the more proximal cause leading to organ failure. In addition, we examined the earlier post-injury development of inhibitory MØ derived mediators and loss of MØ antigen presenting cells (APC) as causes for loss of T cell function in the PBMC.

Input from our clinical co-investigator, Dr. Juan Carlos Puyana, has resulted in adoption of an objective clinical scoring system (the Marshall's multiple organ dysfunction syndrome [MODS] score) for assessing and correlating the onset of clinical complications to MØ and T cell dysfunctions. This scoring system, based on objective physiological parameters derived from the patients' ongoing chart at the time of laboratory measurements, allows a more comprehensive and time relevant appraisal of clinical parameters of MODS which can then be used to indicate which immune aberrations are associated with pathology and which are the coincidental or even beneficial responses to severe trauma

Combining both the use of the MODS score as a quantitative measurement of clinical outcome and evaluating both isolated T cell and PBMC proliferation, we found that although 60-65% of trauma patients (ISS >15) developed depressed PBMC mitogen response at >5 days post-injury, only 30-35% of these went on to develop intrinsic T cell defects (Fig.1). It was this 30% of patients who developed intrinsic T cell defects, who also demonstrated increasing MODS scores. As patient data accrued, it became apparent that patients could be categorized based on their T cell responses (Fig.1). The 35-40% of trauma patients in our studies who showed normal or elevated T cell proliferation in both their PBMC and purified T cell population we have termed phase I type (Appendix A's Fig.1). The 60-65% who developed depressed mitogen induced PBMC T cell proliferation but retained normal or elevated (activated) isolated T cell proliferation were termed phase II patients (Appendix A's Fig.2), while those who progressed to development of a T cell dysfunction in their isolated T cells (intrinsic defect) were termed phase III patients (Appendix A's Fig.3 & 4). The 60% who had depressed PBMC mitogen responses but intact T cell responses (phase II) also had increased production of MØ PGE_2 levels in their PBMC, indicating that inhibitory monokines were contributing to the depression of their PBMC T cell responses (Appendix A's

Table 2). Since this MØ dysfunction did not always correlate with development of MODS, it was not in itself sufficient to cause a negative clinical outcome.

MØ dysfunctions occur in trauma patients and correlate to the development of MODS:

During the initial contract period, we concentrated on characterizing the development of MØ inhibitory monokines which could suppress T cell function, delineating the decrease in MØ/T cell inducing capacity, and defining the loss of MØ endogenous regulatory activities that lead to overexpression of inflammatory cytokines, such as TNF_{α}, which are the mediators of MODS. Depression of T cell proliferation in patient PBMC population and depression of T cell IL-2 and interferon gamma (IFN_{γ}) production were already well characterized defects after severe trauma (9-13).

We have shown that production of the immunosuppressive mediator, PGE_2, was increased post-injury in patients categorized as phase II on the basis of the PBMC mitogen depression (Appendix A's Table 2). However, PBMC from patients in phase III did not show excessive MØ PGE_2 levels, even though their T cell responses were depressed (Appendix A's Table 2). This led us to examine MØ production of another immunosuppressive protein, transforming growth factor ß (TGF_{β}). We published that TGF_{β} was also massively increased in the MØ from trauma patients (14).

The patient MØ overproduction of PGE_2 and TGF_{β} was obviously contributing to the depressed T cell proliferation and activation. However, we had previously shown that addition of the PGE_2 inhibitor, indomethacin, only partially restored patients' T cell responses and only in phase II patients. Consequently, we also examined the production of IL-12 by MØ. IL-12 is critically important in induction and activation of T cells from the Th0 to the T helper 1 (Th1) pathway (15). MØ production of IL-12 was significantly decreased in trauma patients who were exhibiting both the phase II and the phase III type of depressed T cell responses. However, in phase III patients, MØ IL-12 production was almost zero (Fig.2). Monokines are also critical in the generation and activation of Th2 cells. We have recently shown that MØ are critical for maximal production of the Th2 cytokine IL-13 (Fig.3). We were initiating a system to assess patient MØ for their augmentation of IL-13 at the termination of the contract.

In summary, MØ costimulation of T cells, a required step in T cell activation, is impaired in some phase II patients and in all phase III patients. Both TGF_{β} and PGE_2 are also inhibitory to MØ inflammatory cytokine production. The presence of both high MØ PGE_2 and TGF_{β} production

concomitant to excessive MØ TNF_α levels presented a dilemma. How could MØ TNF_α be produced in excess in the face of such high levels of MØ PGE_2 and TGF_β, both of which are downregulators of normal inflammatory monokine production? We found that trauma patients' MØ produced TNF_α that was insensitive to downregulation by PGE_2 and was augmented by TGF_β (see Midterm, Appendix B's Fig.1 and Table 1) (16). The patients who developed MODS also produced primarily cell-associated or $mTNF_\alpha$ (Table II). These data were the first to indicate that MØ production of TNF_α might be aberrantly activated to allow escape from normal MØ endogenous regulatory mechanisms. We also have shown that trauma patients' MØ have exaggerated and prolonged production of TNF_α due to increased TNF_α mRNA stability (see Midterm, Appendix C and Fig.4). Further support for the deregulation of monokine production after trauma in the subset of patients who progress to MODS was provided by demonstrating that MØ stimulated through their Fc receptor produced primarily $mTNF_\alpha$. Several recent published results have increased the significance of our findings. First, it has now been shown that stimulation of MØ through their $Fc_\gamma RI$ receptor in the absence of other induction signals can activate only a limited array of signal transduction pathways and not the wide range of pathways induced by LPS, IFN_γ plus LPS, or other complete bacterial stimuli (17). This means that many monocyte genes normally induced when MØ are stimulated by LPS are not activated in situations where a trauma generated mediator like C3 or Ig complexes are the primary or only MØ stimulator. Second, it has been shown that highly activated MØ shed their LPS receptor making them insensitive to this important multiple induction signal (18). Third, LPS + IFN_γ combine to signal apoptotic elimination of highly activated inflammatory macrophage (19, 20). Since post-trauma MØ lose their LPS receptor (CD14) and are in an environment where IFN_γ levels are depressed, there is greatly diminished post-trauma elimination of these highly activated monokine producing MØ. Finally, $mTNF_\alpha$ has been shown to be more pathologic for normal tissue than is secreted TNF_α (21). Consequently, the post-injury shift to MØ production of $mTNF_\alpha$ indicates increased risk of pathology to normal organs. We have shown that trauma patients whose MØ demonstrate increased $mTNF_\alpha$ also have decreased MØ shedding of their soluble TNF receptors (sTNFR), so that both their increased production of $mTNF_\alpha$ is more pathologic and the neutralization of this $mTNF_\alpha$ is reduced since less TNF_α is bound to neutralizing sTNFR (3). When the MØ aberration in $mTNF_\alpha$ and TNFR is combined, as indicated by a ratio of $mTNF_\alpha$/shed TNFR >100, the patient's risk of mortality is 80% and

there is a high correlation to MODS (Fig.5). These data demonstrate that aberrant monokine production and deregulation of these inflammatory monokines are connected to development of MODS. In addition, we have shown that the deregulated production of TNF_α also correlates to the loss of MØ production of IL-10 (Appendix D's Table VI).

Loss of MØ IL-10 production not only diminishes the downregulation of TNF_α production but also affects the levels of sTNFR shedding. Besides downregulating MØ TNF_α at the mRNA level, IL-10 is also a major stimulation for the shedding of the TNFR (22). Consequently, patients' MØ with reduced IL-10 also have depressed production of the major MØ TNFR (75k), as can be seen in Table III. This loss of MØ TNFR shedding is most evident in patients who also have an intrinsic T cell defect (phase III) (Table III). This is one of the first indications that a loss of T cell responsiveness and the MØ aberrations might both be required, since, although phase II patients (normal/elevated isolated T cell function) showed depressed MØ IL-10, their MØ TNF_α receptor shedding was normal. Since IL-10 is also made by T cells, as long as T cells were not intrinsically dysfunctional, it appeared that sTNFR shedding was proceeding normally. IFN_γ, the product of Th1 cells, is also a potent inducer of TNFR shedding (5).

In summary, then, MØ can be aberrantly stimulated by trauma generated mediators to produce increased amounts of TNF_α which is primarily of the more pathologic $mTNF_\alpha$ type (Appendix E). These aberrantly stimulated MØ produce both increased PGE_2 and increased TGF_β. Concomitantly, the TNF_α produced by these partially activated MØ is not downregulated by auto produced TGF_β, or PGE_2 and the endogenous downregulation by MØ IL-10 is lost because the patients' MØ fail to produce IL-10. The neutralizing effect of shed TNFR is also abated. This leads to a highly activated MØ which has lost its ability to endogenously control its own inflammatory monokine production. However, as long as the trauma patients only developed phase II type immune suppression (i.e. depressed PBMC responses due to inhibitory monokines, but normal/elevated T cell proliferation when isolated T cells were directly stimulated), they did not progress to full MODS. T cell produced IL-10, IL-4, and IFN_γ seem to be controlling MØ inflammatory monokine levels in the phase II patient.

In our examination of patients' MØ dysfunction, we assessed whether patients with excessive TNF_α production were insensitized to IL-10 downregulation as well as lacking in IL-10 production. It might be expected that the patients' MØ were not responding to T cell produced IL-10. We found that

patients' MØ TNF_α levels could still be downregulated by IL-10 (Table IV, Appendix D). Since IL-10 production by T lymphocytes had been suggested as increased post-trauma, it was somewhat surprising to see that excessive trauma patients' TNF_α could be downregulated by exogenous IL-10. In fact, we have shown that another T cell lymphokine, IL-4, could also downregulate both excessive post-trauma PGE_2, TGF_β, and TNF_α production by patients' MØ (23, 24). These data implied that loss of T cell lymphokine production or exogenous control of inflammatory monokines must also be occurring in trauma in addition to depressed T cell proliferation in order for patients to progress to MODS. Since we had already shown that MØ activation of IFN_γ producing Th1 and possibly Th2 cells was decreased, we proceeded to evaluate the full spectrum of lymphokine production in post-trauma Th1 and Th2 cells.

Post-trauma T cell dysfunctions are pivotal in development of MODS:

As already discussed, only those patients whose monocytes are demonstrating deregulated production of the inflammatory monokines are experiencing MODS. However, only the phase III subset of patients who developed an intrinsic T cell defect (failure to proliferate to αCD3/αCD4) concomitantly had MØ whose inflammatory monokine production was deregulated (8). These data indicated that alterations in T cell functions as well as MØ functions were necessary to produce MODS. Involvement of both T cells and MØ in the development of MODS may be one reason why single therapy addressing only excessive monokine production have been generally unsuccessful.

Both Th1 and Th2 type lymphokines are depressed in post-trauma anergy:

The isolated T cell dysfunction (failure of TCR stimulated proliferation) might have reflected increased inhibitory T cell lymphokine production, apoptosis of T cells during *in vitro* stimulation, and/or development of T cell anergy. Stimulation through the T cell receptor plus anti-CD4 should induce proliferation and lymphokine production in the Th0, Th1, and Th2 populations (25, 26). However, excessive IL-10 levels can suppress proliferation of all three T cell subsets if present before T cell stimulation (25, 27). IL-10 is normally made after T cell induction and proliferation, subsequent to IL-2, IFN_γ, and IL-4 (27). We, therefore, examined the cytokine profile of the patients' nonproliferating T cells to determine if high levels of IL-10 were initially present. In the phase II patients' PBMC, IFN_γ, IL-2, IL-4, and IL-10 were all depressed (Table V). These data indicate both that increased IL-10 was not responsible for the depressed T cell proliferative response and also that MØ inhibitory factors, rather than

lymphokines, are reducing PBMC T cell lymphokine levels probably by depressing T cell proliferation. This data interpretation is supported since phase II patients' isolated T cells showed normal to elevated proliferation and their IFN_γ, IL-2, IL-4, and IL-10 were also concomitantly normal/elevated (Table V). Although IL-10 was often elevated after stimulation of the phase II isolated T cells, it was not inhibiting T cell proliferation, presumably because it was being produced subsequent to proliferation. It also appeared that elevated post-trauma T cell IL-10 was critical to exogenous control MØ inflammatory cytokine production. This simultaneous induction of both Th1 (IFN_γ) and Th2 (IL-4) lymphokines also indicates that phase II patient T cell populations consist of either both effector T cell subsets or mostly Th0 type. Most human T cell responses are initially of the Th0 type (25). Since IL-2, IL-10, and IL-13 are made by all three human T cell subsets, albeit in different amounts, detection of these lymphokines does not indicate a predominance of one subset (25, 27). In striking contrast to the phase II results, the phase III, non proliferative or anergic patients' T cells had depressed IL-2, IL-4, IFN_γ, IL-10, and IL-13 (Table V & Fig.6). These data indicate that the phase III patients' T cell unresponsiveness is not due to inhibitory lymphokine action of IL-10 or T cell predominance of Th2 type lymphokines, like IL-4 or IL-13. Increased shedding of IL-2 receptor α chain (IL-2R, CD25) is reported as increased in burn patients' T cells (28). Thus, our phase III patients' T cell unresponsiveness could result from increased shedding of IL-2R and failure to respond to IL-2. We found that IL-2R levels were actually elevated in T cell supernates from phase II patients, concomitant to their normal/elevated proliferation but dramatically depressed in phase III patients' unresponsive anergic T cells and that IL-2R levels were normal in unresponsive patients' T cells (Fig.7). Thus, phase III patients' T cell unresponsiveness is not due to an increased IL-2R shedding. Most importantly, the loss of regulatory type II lymphokines, like IL-4, IL-10, and IL-13, correlate to aberrant production of monokines, as indicated by increased production of $mTNF_\alpha$ (Fig.8 & Table V). The loss of Th1 and Th2 lymphokines also correlate to MODS (Tables V & VI). Both the Th1 and Th2 were equally anergic in the trauma patients' T cell population. This is demonstrated by the partial restoration of both Th1 and Th2 lymphokine levels when IL-2 is exogenously added to phase III patient T cells (Fig.9). One definition of T cell anergy is the failure of activated T cells to proliferate or produce IL-2 but to be partially restored by exogenous IL-2 addition (29). We, therefore, assessed the effects of exogenous IL-2 addition on patients' anergic T cells. If only Th1 or only Th2 T cells were

unresponsive, then restoration of proliferation by IL-2 should have led only to restored IFN_γ or IL-4. Restoration of IFN_γ and IL-4, as well as IL-10, demonstrate that anergy effects both Th1 and Th2 cells (Fig.9). The development of anergy in the Th1 and Th2 lymphocyte population correlates to development of MODS and to deregulated production of TNF_α, as indicated by a $mTNF_\alpha/sTNF_\alpha$ ratio of >100 (Tables V & VI). These data again link the loss of T cell lymphokine production to aberrant monokine responses. Loss of T cell production of IFN_γ leads to failure to appropriately trigger the whole array of MØ genes, including cytokine regulatory genes, as well as reduced apoptotic MØ elimination. Failure to maintain high levels of T cell IL-4, IL-10, and IL-13 post-trauma, leads to loss of exogenous downregulation of inflammatory monokine production.

Post-trauma anergy and apoptosis are not occurring concomitantly:

It was also possible that the phase III patients' T cells were undergoing accelerated activation induced cell death (AICD), eliminating these activated T cells, to produce unresponsiveness and loss of lymphokine production. The isolated phase III T cells were examined for viability in three ways. First, apoptosis was directly assessed by flow cytometry, as described, using propidium iodine (PI) from fixed, permeabilized, MØ depleted PBMC (Fig.10) (30). When all trauma patients were examined as a group, the patients had higher levels of apoptosis than normals (Fig.11). However, the phase III unresponsive T cells had less apoptosis than PHA stimulated normals, indicating that these T cells were not apoptotic (Table VII). This is not surprising since production of IL-2 is required for AICD and IL-2 is depressed in these patients' T cells (Table VII) (31). In contrast, the highly proliferating phase II patients' T cells exhibited increased apoptosis, indicating AICD was proceeding normally in this proliferating population (Fig.10, Fig.11). In a second test for viability, phase III T cells were examined by quantitative Mimic PCR for levels of the housekeeping gene G3PDH at 6 hr post-culture (a time when apoptosis should be high). As can be seen in Figure 12, the unresponsive phase III and normals' T cells made identical amounts of G3PDH after 6 hr of culture, indicating the anergic T cells were viable and that lymphokine production was selectively decreased. Finally, the phase III T cells were cultured with anti-CD3/anti-CD4 for 72 hr, then the cells were stained with trypan blue. Unresponsive phase III T cells from 10 patients were assessed versus T cells from similarly stimulated normals. Both normals' and phase III patients' T

cells were >90% viable after the 48 hr culture, indicating the patients' T cell failure to respond was not due to cell death.

Post-trauma anergy might be related to depressed lymphokine stimulation:

These data indicate that phase III unresponsive T cells are anergic. The definition of T cell anergy is loss of proliferation and IL-2 production subsequent to a productive response (32). Anergic human T cells can be restored by exogenous IL-2 addition but lose their ability to respond to IL-12 (29). As can be seen in Figure 9, the proliferative response of phase III patients' T cells could be partially restored by addition of 100U/ml of exogenous IL-2. In addition, IFN_γ, IL-4, and IL-10 responses were also partially restored upon exogenous IL-2 addition (Fig.9). These data indicate that the phase III patients' T cells are anergic and represent either a Th0 population or a mixed Th1 and Th2 population. As further evidence of the anergic state of the patients' phase III T cells, we have examined the effect of exogenous IL-12 addition on the T cell proliferation and IFN_γ production of T cells from phase III versus phase II patients, since anergic T cells lose their responsiveness to IL-12 (29). As can be seen in Appendix F's Fig.4, IL-12 addition could reverse the proliferative depression in PBMC from phase II patients, again supporting the concept that MØ dysfunctions, not a T cell defect, is operative in depressing the T cell mitogen responses of these patients. In contrast, addition of IL-12 had no restorative effect on PBMC proliferation in phase III patients (Appendix F's Fig.5). The IFN_γ production of isolated phase II patient T cells ($\alpha CD3/\alpha CD4$ stimulation) is normal or elevated while that of phase III is depressed. IL-12 addition to isolated phase III patient T cells also does not restore IFN_γ production (Table VIII), illustrating that their T cells' defect in IFN_γ production was an intrinsic defect and consistent with T cell anergy. The development of T cell anergy as indicated by loss of proliferative function and IL-2, IL-4, IL-10, and IFN_γ production has serious consequences for the trauma patients. Only development of the T cell anergy correlates to increasing MODS scores (Table V). Part of the IL-12 enhancing effect on T cell IFN_γ production operates by boosting the mRNA translation level (33). Interestingly, IL-12 had a moderate restorative effect on phase III T cell IFN_γ mRNA levels (Fig.13), although not restoring them to normal mRNA levels. Nevertheless, no similarly enhanced IFN_γ protein production was produced (Table VIII). These data illustrate that an IL-12 signal is received by the anergic T cells through their IL-12 receptor but the secondary signal transduction for accelerated IFN_γ mRNA translation is blocked (Fig.14). These data

indicate that both the MØ production of IL-12 and the ability of t cells to respond to IL-12 is depressed in patients who proceed to MODS.

Phase III anergic T lymphocytes have an intrinsic proliferation defect, depressed lymphokine production and are unable to completely respond to IL-12. Phase III patients' T cells also are unable to respond to PMA, indicating a global T cell defect (Table IX). Phase III anergic T cells seemed unable to respond to a second proliferative stimulus possibly because a negative signal was received due to incomplete activation of the TCR. One possibility was that downregulation of CD3 on patients' T cells was resulting in incomplete antigen signaling, thereby allowing negative signaling and preventing any further induction of anergic T cells. We examined the CD3 expression on the patients' anergic T cells. As seen in Figure 15, the level of CD3 is decreased in the anergic patients' T cells versus the T cells from normal or phase II (non anergic) patients. The loss of CD3 does not explain these anergic cells' failure to proliferate to PMA nor does it explain why both IL-2 and IL-4, along with IL-10 and IFN_{γ}, are still depressed after PMA stimulation. The loss of another surface stimulatory ligand may contribute to this loss of critical growth sustaining and monokine regulatory lymphokines or to negative signaling. Human autoreactive T cells and "suppressive" T cells have been described as expressing CD11b, the receptor for the third component of complement (34). Although most T cells are CD11b negative or low, expression of CD11b increases in some autoreactive T cells with restricted response capacity (34, 35). Additionally, CD11b is only expressed on T lymphocytes as CD28 expression is downregulated (34). Since CD28 costimulation is absolutely required both for maximal IL-2 and IL-4 production, we examined both CD28 and CD11b expression on the phase II (non anergic) versus phase III (anergic) patients' T cells (36, 37). As can be seen in Figure 16, as patients' CD11b expression increased, T cell proliferation decreased. Additionally, as CD28 expression decreased, CD11b expression increased (Fig.17). Decreased CD28 on anergic T cells would drastically reduce IL-2 and IL-4 production and continued T cell proliferation (36). Since Th2 IL-4 activation seems to be a prerequisite for adequate IL-10 production, reduced IL-4 production would also result in our observed reduced IL-10 levels (38). Finally, we have shown that decrease in CD3 expression, concomitant to increased CD11b expression, parallels both increasing MODS scores and decreased proliferation (Fig.18). These data indicate that the anergic T cells have aberrant receptor expression indicative of the alterations in some of their activation functions. One possibility

which needs to be further explored is that failure of MØ to deliver appropriate costimulatory signals is pivotal in the development of this post-trauma T cell anergy.

Excessive apoptosis during anergy development may intensify post-trauma T cell dysfunction:

The loss of CD28 can also result in increased apoptosis so we have also examined the segregated (panned) $CD11b^+$ anergic T cells for increased apoptosis (39, 40). However, in the initial two experiments, there did not appear to be increased apoptosis in the population that is $CD11b^+$. There is increased apoptosis in overall trauma patients' T cells (phase I, II, & III). However, if the phase III trauma patients' T cells are separately assessed for apoptosis using flow cytometry and PI staining, the anergic T cell population has lower levels of apoptosis after induction than normal individuals' similarly stimulated T cells (Table VII). In contrast, phase II patients' T cells have increased T cell apoptosis over normals' (Table VII). The loss of T cell apoptosis, like the loss of T cell proliferation, corresponds to increasing MODS scores (Fig.19). These data demonstrate that at the point of maximal T cell anergy the patients' T cells are not apoptotic. In addition, the phase II patients' apoptosis seem to be mediated through the Fas/FasL system (Fig.11). It is not surprising that a high level of Fas mediated apoptosis accompanies the high levels of T cell activation seen in phase II patients.

A small number of patients who we have repeatedly assessed over their post-injury period showed a period of overlap when their T cells were becoming anergic but remained highly apoptotic. (One such patient is illustrated in Fig.20.) There are multiple interpretations of these data. The high level of apoptosis present during early anergy could just reflect that apoptotic function is less dependent on lymphokine production and, therefore, persists during initial lymphokine depression. In this case, expression of Fas on the T cell surface may be only slowly downregulated as the T cells become anergic. In addition, the protective effect of shedding sFas may rapidly disappear before the loss of T cell surface Fas, transiently increasing the patients' apoptosis. Alternatively, the patients' early anergic T cell population may contain both anergic and responsive T cells. The responsive T cells may become transiently sensitive to a secondary non Fas mediated apoptotic pathway like TNF, which augments the degree of apoptosis, eliminating reactive T cells and leaving only anergic cells (41). We have shown that the phase II patients' T cells have increased sensitivity to anti-Fas, suggesting again that phase II apoptosis is appropriately Fas mediated (Fig.11). Increased Fas mediated apoptosis of activated T cells during

surgical stress has already been demonstrated (42). However, our data would indicate that this Fas mediated apoptosis acts to appropriately control highly activated post-trauma lymphocytes. It is the development of T cell anergy and loss of apoptosis that presages the development of MODS.

In summary, our data show that a subset of patients develop T cell anergy which includes both Th1 and Th2 type cytokines. We assessed the clinical relevance of alterations in T cell function and lymphokine production by correlating them to the development of multiple organ dysfunction and consequent mortality, as indicated by increasing MODS scores. IL-2 addition partially restored both IL-4 and IFN_γ levels, again suggesting that both Th1 and Th2 cells are anergic in the phase III trauma patients. We have shown lymphokine depression in the anergic T cells at the protein (ELISA & Western) and at the mRNA levels using Mimic PCR. A defect in post trauma T cell stimulation and activation may be linked to decreased post-trauma CD3, decreased CD28, and/or an increase in an inhibitory signal delivered by C3 split products through upregulated CD11b. In addition, IL-12 is able to signal anergic T cells for IFN_γ mRNA increases but not for IFN_γ protein increases, possibly indicating a partial blockade of T cell signal pathways. Finally, we have examined apoptosis with flow cytometry and shown a loss of apoptosis at the height of anergy but perhaps increased apoptosis at the initiation of anergy. We have delineated T cell alteration in relationship to the onset of organ dysfunction in order to identify both possible T cell targets for combination therapy and an optimal time period for this therapy.

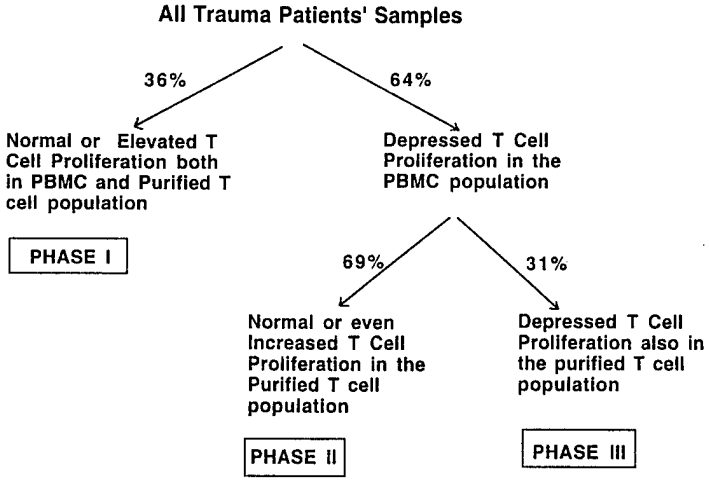
CONCLUSIONS:

In conclusion, we have shown that the subset of trauma patients who develop MODS, as indicated by having a Marshall's MODS scores >6, have both MØ and T cell dysfunction (Fig.21). Initially, post-injury, both MØ and T cells are appropriately activated. Trauma patients whose MØ are producing primarily secreted TNF_α, are also producing some immunosuppressive monokines, such as PGE_2 and TGF_β which, along with accelerated shedding of the TNFR, control their TNF_α levels. In addition, T lymphocytes are highly activated on these patients, as evidenced by their increased responsiveness when stimulated directly through the TCR. T cells produce high levels of IFN_γ, IL-4, IL-10, and IL-13, controlling monokine production. However, production of inhibitory MØ mediators, such as PGE_2, also

controls the activation of these trauma induced T cells, resulting in a balance between inflammatory and immune system activation. In addition, T cell apoptosis, through AICD is increased and eliminates these trauma hyperactivated T cells. This profile is typical of the phase II patient and, though very different from a "normal", seems to be an appropriate post-injury response, leading to control of infectious challenge and eventual recovery. In marked contrast, a small proportion of trauma patients develop both a MØ and T cell dysfunction and proceed to develop MODS. The MØ from these phase III patients are producing high levels of proinflammatory monokines, such as TNF_{α}. The phase III patients' MØ production of TNF_{α} is deregulated and aberrant, as indicated by increased TNF_{α} mRNA stability, failure to shed neutralizing TNFR, insensitivity to PGE_2 and TGF_{β} downregulation, as well as a predominant production of $mTNF_{\alpha}$. The ability of these aberrant post-injury MØ to appropriately activate T cells is also decreased, as indicated by loss of their IL-12 production. In concert with this severe post-trauma MØ dysfunction, both Th1 and Th2 T cell responses are depressed. The phase III patients' dysfunctional T cells can no longer appropriately activate or regulate inflammatory MØ and allow exaggerated inflammatory monokines to cause MODS. Loss of MØ function is detectable as a change in the MØ surface receptor ligand expression. There is an increase of MØ TNFR expression levels due to failure to shed the TNFR. There is aberrant expression of $mTNF_{\alpha}$ and the loss of HLADQ expression. Aberrant T lymphocyte activity is also rapidly indicated by a persistent loss of CD28 and CD3 expression with a concomitant upregulation of CD11b expression. Rapid flow cytometric identification of these altered MØ and T cell surface receptor/ligand combinations might serve as an easily implementable technique for screening combat casualties. The data from this contract have implicated a combination of T lymphocyte and MØ dysfunctions as responsible for the development of MODS. Both a possible means of rapidly identifying combat casualties at risk of MODS and suggestions of future interventive therapy have been developed as a result of this contract support.

Summary of the T cell proliferative (both in the PBMC and isolated T cell population) response pattern/phase of all the 98 samples collected from 40 patients at different post-injury days.

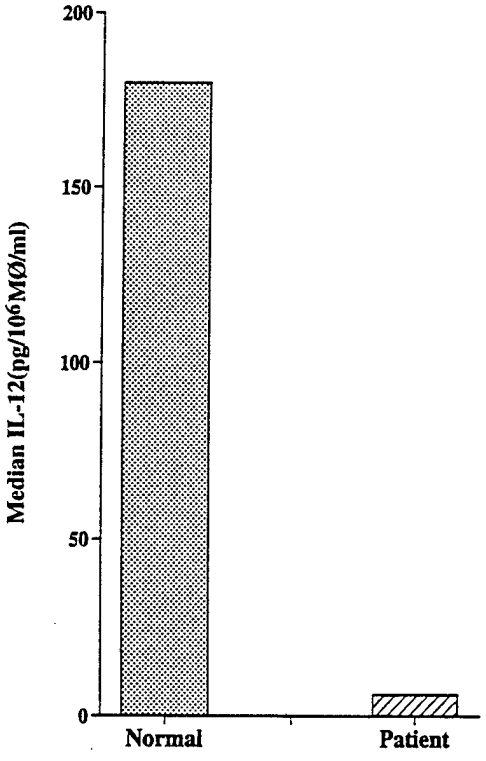
Figure 1



PBMC and purified T cells (2 x 10^5 cells/200µl/well) were assessed for proliferation (^{3}H-thymidine uptake) in response to PHA (1µg/ml) and immobilized anti-CD3 (1µg/well) + anti-CD4 (1µg/well) respectively in 72 h of culture. When the proliferation of PBMC or purified T cells is at least 30% less than that of the paired normal, it is considered depressed.

Figure 2

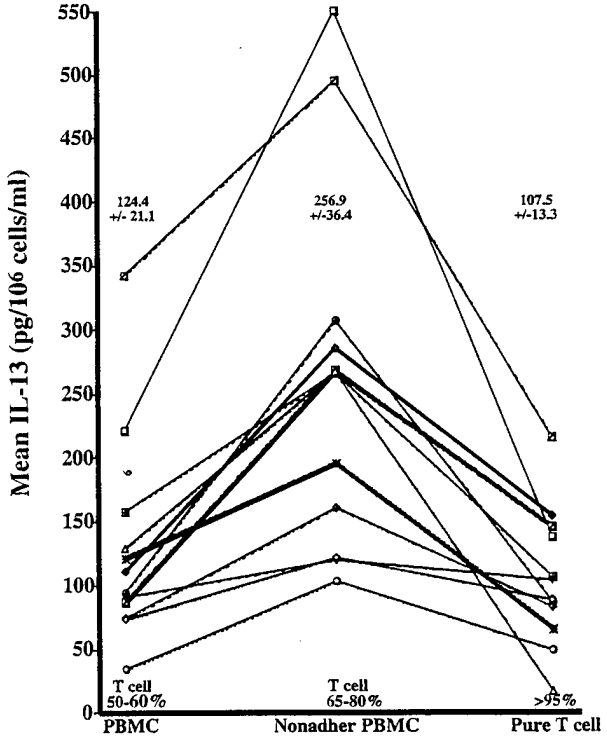
Depressed IL-12 Production by MØ in Phase III Patients



MØ cultured(3x10^6 MØ/3ml/well)for 16 hrs were assessed for IL-12 production by ELISA.

Figure 3

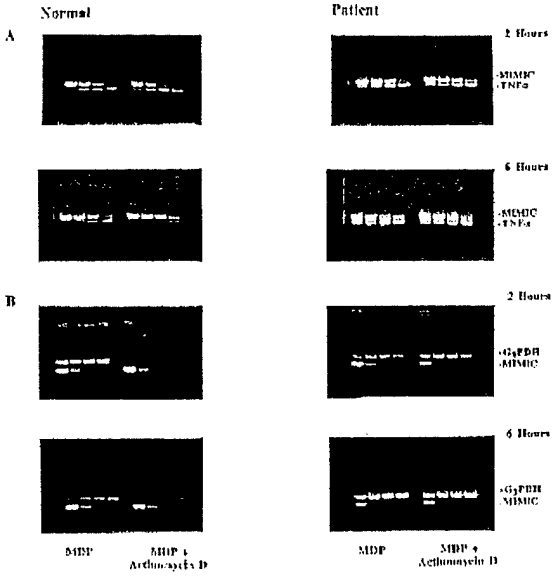
Maximal IL-13 Production Requires An Accessory Non T cell



Peripheral blood mononuclear cells (PBMC), microexudate-coated plastic non-adherent PBMC and E-rosetted T cells from normals (n=14) were cultured (1x10^6 cells/ml) in presence of PHA (1µg/ml) + PMA (10ng/ml) for 20-22 hrs. IL-13 levels in the culture supernates were assessed by ELISA. IL-13 production by non-adherent PBMC was significantly higher than either PBMC (p=.001) or purified T cells (p=.001).

TNFα mRNA is More Stable in Patient MØ

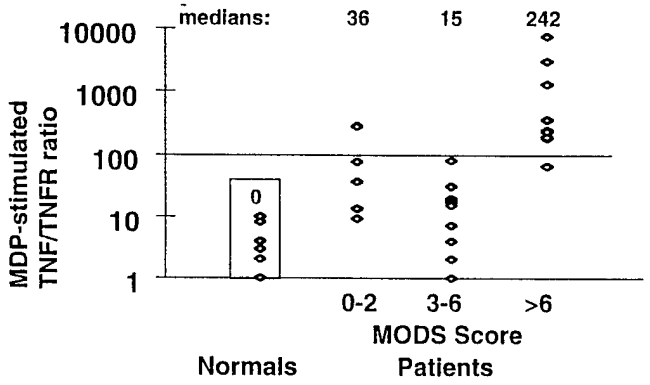
Figure 4



MØ from a hyperaccumulating patient and a normal control were cultured for two or six hours with or without addition of MDP. At two hours post induction, 1μg/ml actinomycin D was added to indicated cultures. At indicated times post MDP induction, cells were lysed and RNA was analyzed. A). PCR was carried out using TNFα specific primers and the TNFα Mimic. B). PCR was carried out using G3PDH specific primers and the G3PDH Mimic.

Monocytes develop deregulated TNFα production

Figure 5



For each timepoint that patients' MØ were studied, a mTNF/TNFR ratio was calculated. The patients' peak MØ mTNF/TNFR ratio during their study period is represented along with the MODS score on that day. There is a highly significant difference (p=0.0005) between low, medium, and high MODS score groups. Chi-squared analysis using a cut-off ratio of 100 and MODS ≤6 or >6 is significant at p=0.0002.

Figure 6

Depressed IL-13 levels correlates to anergy development and organ dysfunction

	% MODS score >6	anergy develops
IL-13 < 50% NL	34%	90%
IL-13 > 50% NL	10%	20%

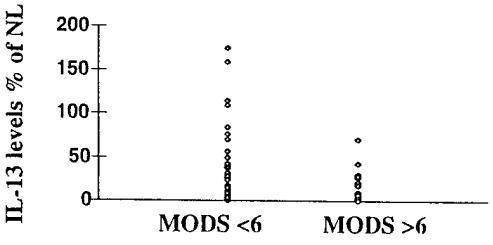
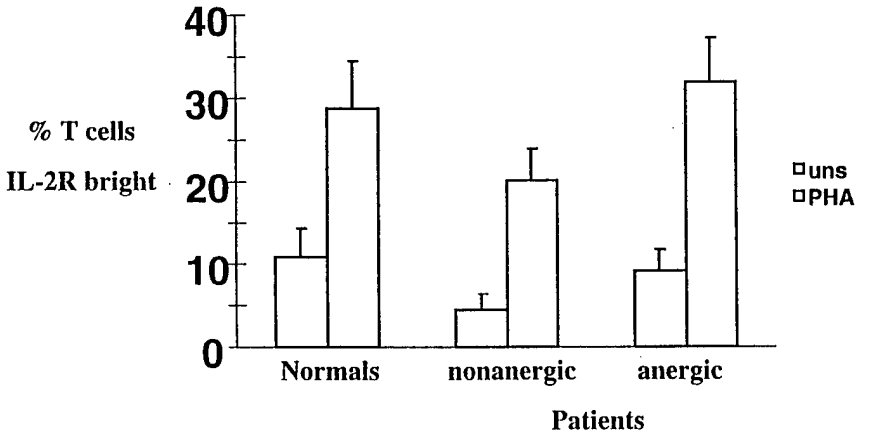


Figure 7

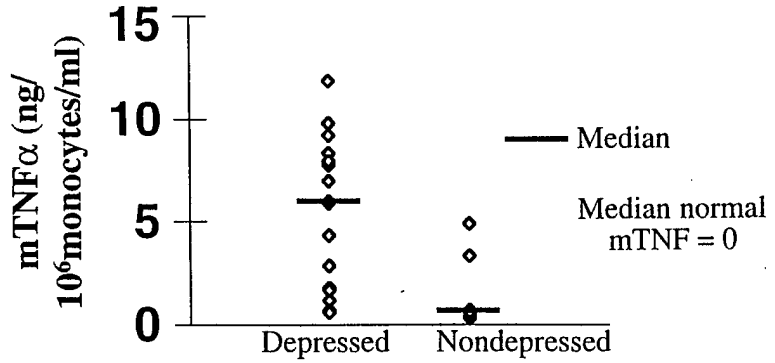
Anergic T cells normally upregulate their IL-2 receptors when stimulated



T cells are analyzed flow cytometrically for IL-12 receptor (IL-2R) or CD25 expression. Results = % of T cells positive for IL-2R expression. There is a statistically significant upregulation of IL-2R with PHA stimulation for all groups (normals, nonanergic, and anergic). There is no significant difference of IL-2R levels between normals and patients.

Figure 8

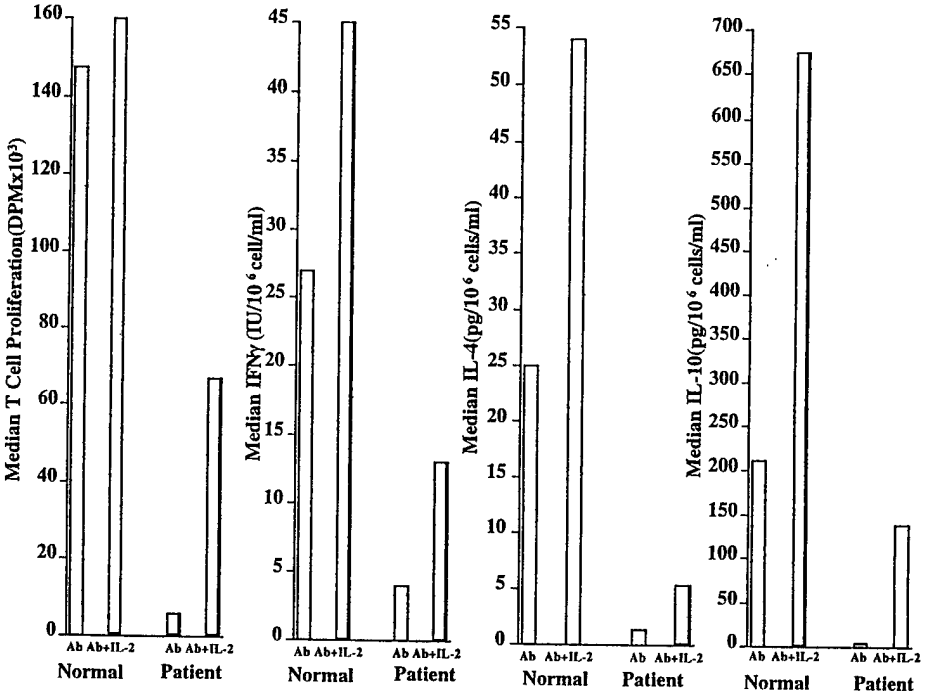
Depressed IL-13 levels correlates to monocyte dysfunction



All patient IL-13 assays were stratified into two groups: those assays where patient T cells produced IL-13<50% of the simultaneous run normal levels are designated depressed. Conversely, those assays in which the patients' T cells produced >50% of the simultaneous run normal are undepressed. IL-13 was measured by ELISA. mTNF was measured in the LM cell bioassay. When patient T cell IL-13 production is decreased, there is a significant ($p<0.05$) increase in their MØ mTNF.

Figure 9

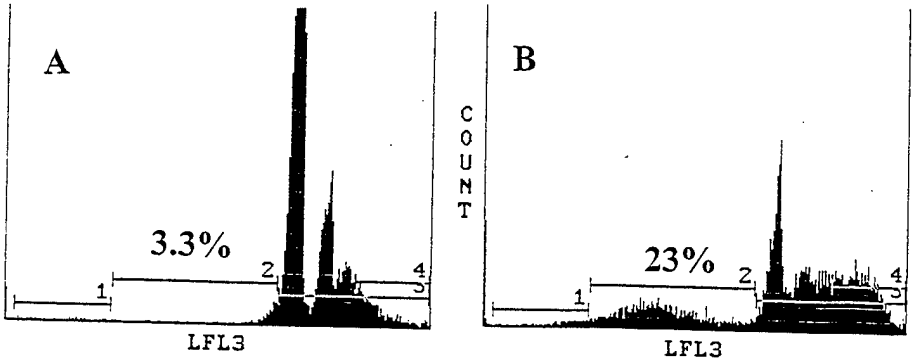
Partial Restoration of Proliferation and Lymphokine Production of Anergic T cells by IL-2



MØ-depleted SRBC-rosette purified T cells from normals and trauma patients were simultaneously assessed for proliferation (72 hr of culture) and different lymphokine production (24 hr of culture) in response to immobilized anti-CD3 (1µg/well) + anti-CD4 (1µg/well) (Ab) alone or Ab+IL-2 (100U/ml).

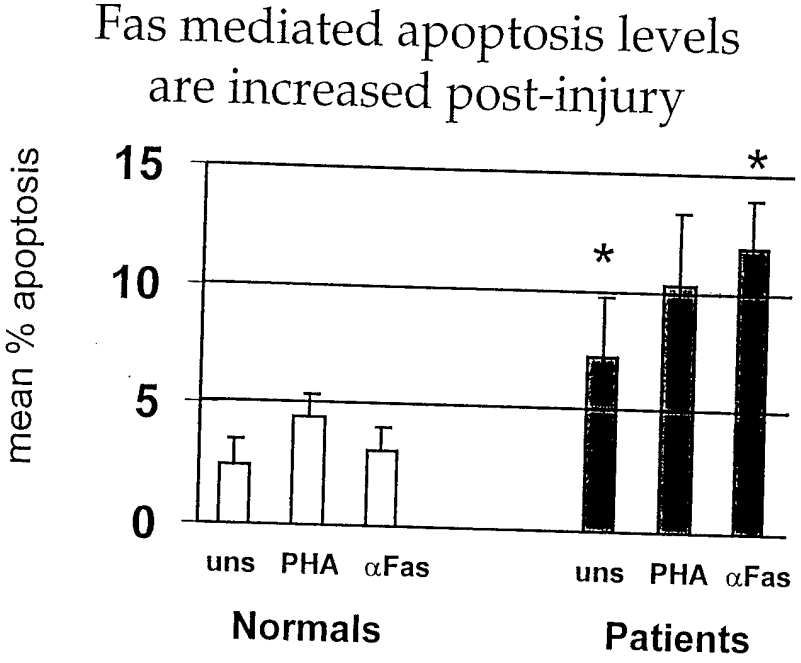
Figure 10

Representative Histograms



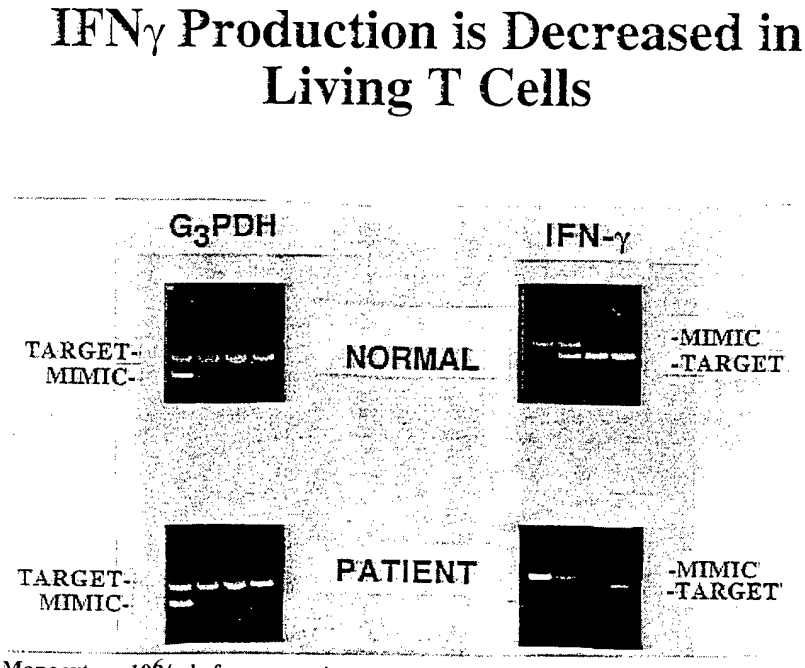
"A" represents a normal's unstimulated T cells which serves as the negative control. LFL3 = the fluorescence intensity of incorporated propidium iodide. Gate 2 represents the subdiploid fraction of cells, which in this case is 3.3%. "B" represents a patient's T cells stimulated with PHA which are undergoing a significant level of apoptosis (23%).

Figure 11



In addition to culturing normals' and patients' monocyte depleted PBMC unstimulated or with PHA stimulation, cultures were also similarly set up with α-Fas mAb (100ng/ml) which would bind Fas and trigger apoptosis. In the normals' cells, there was no significant difference in the levels of apoptosis between the unstimulated and α-Fas stimulated cultures. In contrast, there was significantly increased levels of apoptosis among patient cells when stimulated with α-Fas as compared to no stimulation (* $p < 0.003$).

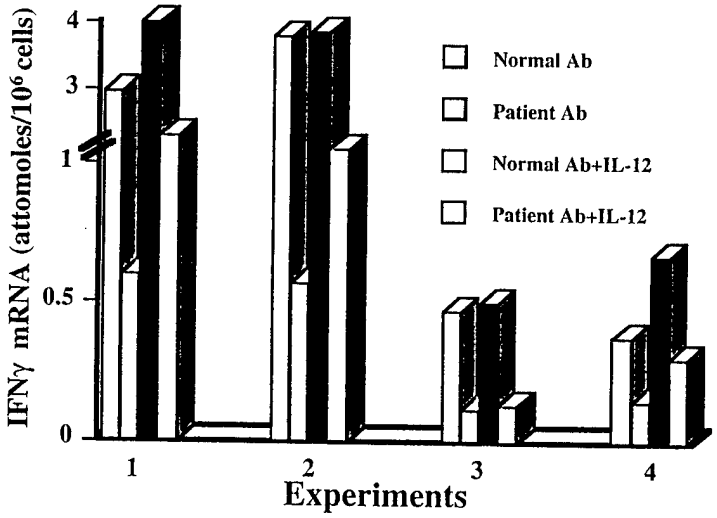
Figure 12



Monocytes, 10^6/ml, from a patient and a normal control were lysed and whole cell RNA was reverse transcribed and used in competitive PCR. Amplification products were electrophoresed through 1.5% agarose TAE gels with 0.1 ug/ml ethidium bromide. A: PCR was carried out using TNFα specific primers and the TNFα Mimic. B: PCR was carried out using G_3PDH specific primers and the G_3PDH.

Figure 13

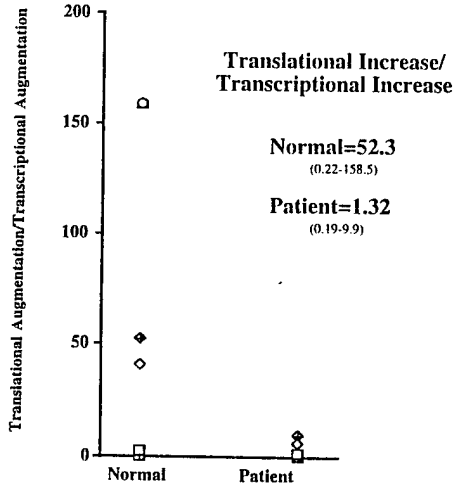
IL-12 Partially Augments IFNγ mRNA Production in Patient T cells



Cells were incubated in wells coated with anti-CD3 and anti-CD4 either without (Ab) or with (Ab+IL-12) the addition of IL-12 to a final concentration of 100U/ml. Cells were lysed and whole cell RNA was reverse transcribed and used in competitive PCR to determine IFNγ specific RNA levels.

Figure 14

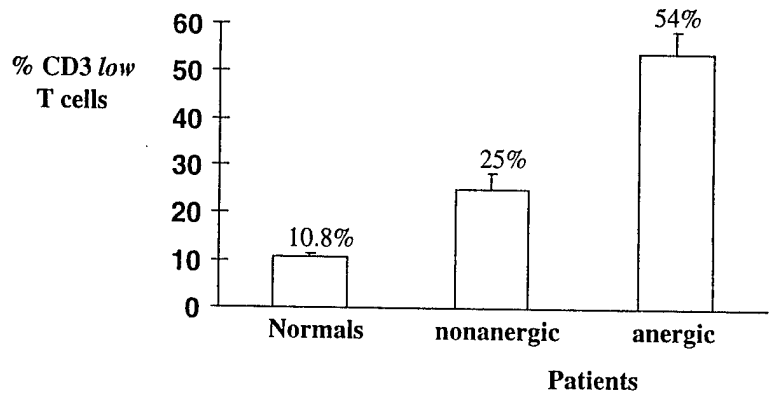
IL-12 Induced Translational Augmentation of IFNγ is Impaired or Absent in Patients' T Cells



Cells were incubated in wells coated with anti-CD3 and anti-CD4 either without (Ab) or (Ab+IL-12) the addition of IL-12 to a final concentration of 100U/ml. IFNγ protein production was determined using ELISA and LM cell assays. Whole cell RNA was reverse transcribed and used in competitive PCR to determine IFNγ specific RNA levels. The increase in amount of IFNγ protein produced by Ab+IL-12 cultured cells compared to Ab cultured cells divided by the increase in the amount of IFNγ specific mRNA content in Ab+IL-12 cultured cells compared to Ab cultured cells.

Figure 15

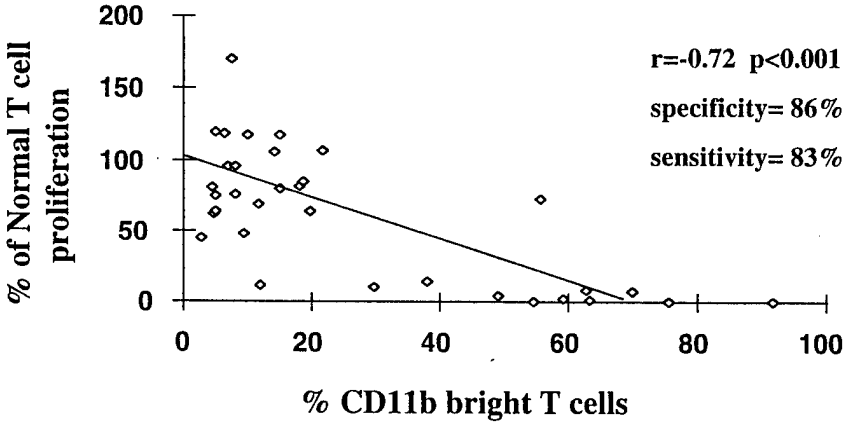
Patients' anergic T cells have downregulated CD3 expression



T cells were flow cytometrically analyzed for CD3 (TCR or T cell receptor) expression using a fluorescein tagged monoclonal Ab. Results = mean percentage of T cells which are CD3 bright + SEM. The means from all three groups are statistically different ($p < 0.05$).

Figure 16

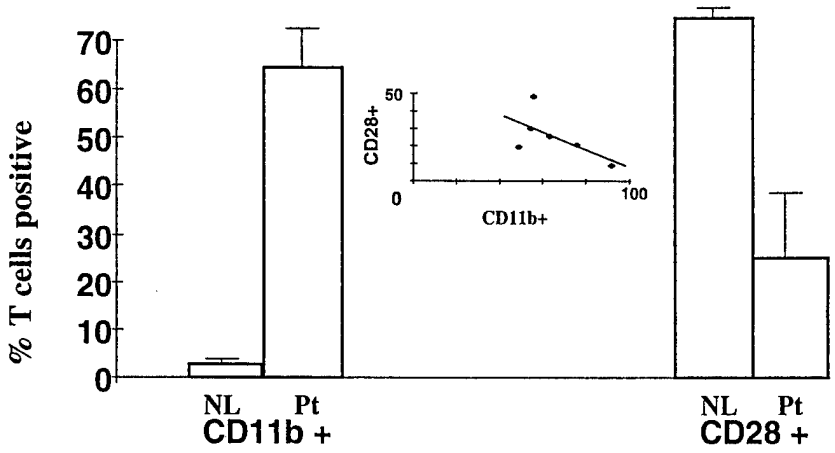
Anergic T cells have upregulated C3b (CD11b) receptor



As anergic T cells' CD11b expression increases, their T cell proliferative capacity to αCD3/αCD4 decreases. Sensitivity and specificity were determined by using > or < 50% of normal T cell proliferation and > or < 20% CD11b bright T cells.

As CD28 is downregulated, the C3b receptor (CD11b) is reciprocally expressed

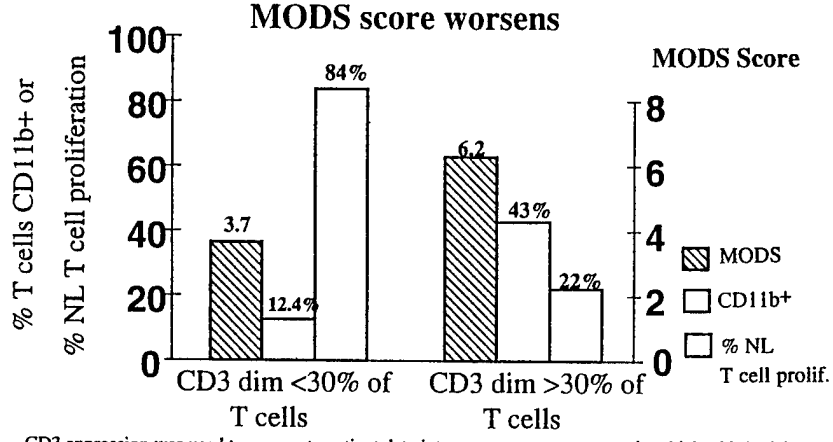
Figure 17



It has been previously shown that CD28 and CD11b (the C3b receptor) expression are reciprocal. Here, we show that patients whose anergic T cells have decreased CD28 expression also have reciprocally increased CD11b expression (flow cytometric data).

Figure 18

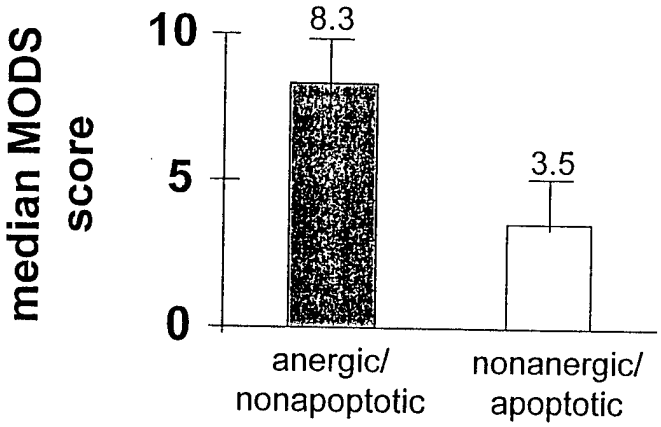
As T cell CD11b expression increases and CD3 expression decreases, T cell function is lost and MODS score worsens



CD3 expression was used to segregate patient data into two groups: one group in which <30% of the T cells had decreased CD3 expression (CD3 dim) and the other group in which >30% of the T cells were CD3 dim. Decreasing CD3 expression (a higher proportion of CD3 dim T cells) correlates to increased CD11b expression (%CD11b+) and worsening MODS score, as well as decreased T cell proliferation to αCD3/αCD4 represented here as % of the simultaneously assessed normal's proliferation.

Level of apoptosis may correlate to patients' outcome

Figure 19



We use the MODS scores only from the days in which the patients' T cells were found to be both anergic and nonapoptotic (apoptosis the same or less than the normals') or functional and apoptotic in response to PHA. When T cells are anergic and nonapoptotic, the MODS score is more than doubled compared to when they are functional and apoptotic ($p<0.005$).

Figure 20

Temporal relationship of apopotosis and anergy

T cells are anergic and nonapoptotic

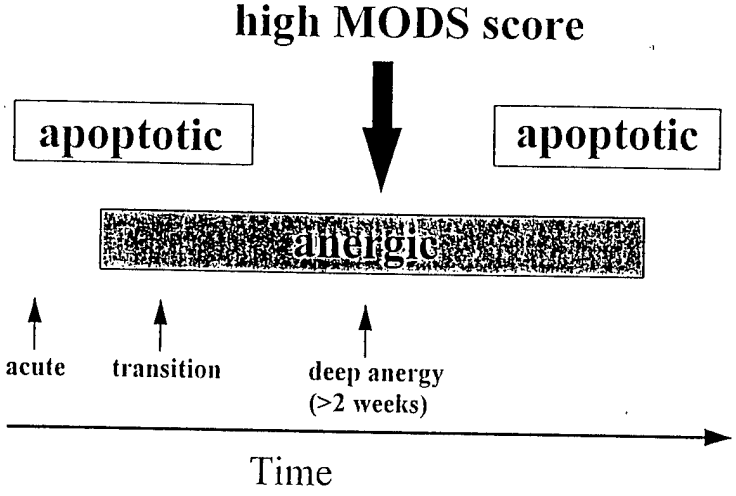


Figure 21

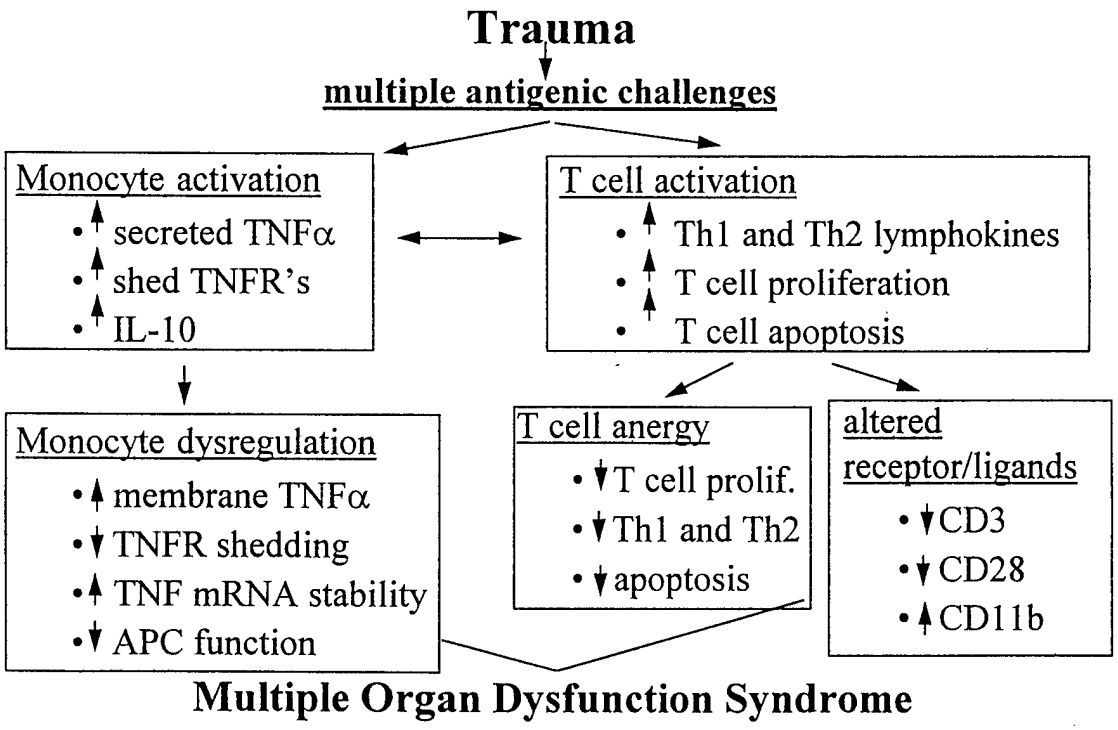


Table I - Summary of PGE_2, TNFα Augmentation, and T Cell Responses to PHA and Antibody

Patient	Injury	PGE_2 [a]	TNFα [b]	PHA [c]	% T Cell Response [d] and Outcome [e]
1	Burn	5.6→ 17.7	44.17	-59%	74→ 126→ 147 - No major complications
2	Burn	8.9→ 18.4	59.8	-73%	89→ 71→ 1→ 25→ 18 - MOF -Expired
3	Burn	32.5	80.36	-91%	27→ 40→ 33→ 50→ 33 - MOF - Expired
4	Trauma	13.4→ 11.5	.87	-23%	80 - No major complications
5	Trauma	2.8→ 5.6	3.27	-22%	48→ 39 - No major complications
6	Trauma	14.6→ 45.9	41.79	-75%	52→ 22→ 117→ 135→ 154 - Major complications
7	Trauma	4.8	<1.0	+121%	80 - No major complications
8	Trauma	4.0→ 9.5	1.49	+140%	40→ 95→ 138→ 109 - No major complications
9	Trauma	3.4→ 4.1	<1.0	+130%	164→ 203→ 61 - No major complications
10	Burn	.46→ 1.32	12.9(0)	-35%	29→ 37→ 84 - No major complications
11	Trauma	.368	0(0)	+67% *	65 - No major complications
12	Trauma	.339→ .982	164.9(0)	-7%	101→ 16 - No major complications
13	Trauma	.107→ 25.7	174.3(0)	-79%	9→ 220→ 97→ 21 - Major complications
14	Trauma	.099	0(0)	-83% *	124 - No major complications
15	Burn	.17→ .44	125.1(0)	-75%	3→ 29→ 138 - No major complications
16	Burn	2.7→ 16.8	84.3(0)	-41%	17→ 73→ 151 - No major complications
17	Burn	17.1	0(0)	-8%	271 - No major complications
18	Burn	- - -	- - -	-64% *	Expired
19	Burn	.487	17.1(0)	-40% *	53 - No major complications
20	Burn	.492→ .694	231.5(7.7)	-83%	7→ 6→ 30 - MOF - Expired
21	Burn	.168→ 2.677	429.2(0)	-69%	40→ 11→ 6 - MOF - Expired
22	Trauma	.6→ 1.247	234.8(0)	-93%	144→ 105→ 6→ 5 - Major complications
23	Trauma	.314	14.9(0)	-98%	No major complications
24	Trauma	.854	0 (0)	-40%	11 - No major complications
25	Trauma	.512→ .567	9.7(0)	-76%	5→ 170→ 97→ 120 - No major complications
26	Burn	6.2	2.7(0)	-21%	248→ 204 - No major complications
27	Trauma	.393→ 4.1	2.5(1.8)	-68%	107 - No major complications
28	Trauma	.187→ 1.62	3.0(.6)	-45%	2→ 23→ 81→ 127 - No major complications
29	Trauma	.5→ 29.3	5.4(1.0)	-62%	156→ 72→ 114→ 63→ 116→ 91→ 63 - No major complications
30	Trauma	- - -	3.8(0)	-63%	147 - No major complications
31	Trauma	6.6→ 24.1	5.1(0)	-14%	176 - No major complications
32	Trauma	.05→ .36	15.9(.98)	-86.8% *	28→ 1.0→ 3.1 - MOF - Expired
33	Trauma	.18→ 1.93	4.1(.7)	-87.1%	165→ 3.8→ 14.5 - Major complications
34	Burn	.42→ 3.81	2.5(2.0)	-76.1% *	12.3 - No major complications
35	Burn	.22→ 3.22	28.5(3.6)	-97.7%	15.6→ 1.0→ 23→ 3.2→ 1.4→ .5→ 4.7→ 4.7→ .2 - MOF - Expired
36	Trauma	.30→ 15.78	34.1(3.6)	-80.3%	7.6→ 112→ 83.1→ 104→ 35.4 - No major complications
37	Burn	.46→ 1.45	30.0(3.6)	-59.9% *	4.9→ 75.5→ 39.5→ 101→ 34.8 - Major complications
38	Burn	1.70→ 11.98	6.1(3.6)	-67.2% *	101→ 62.9→ 95.8→ 43.7 - No major complications
39	Burn	.63→ 1.19	7.8(0)	-69.5%	15.6→ 115.8→ 118.0→ .8 - MOF - Expired
40	Trauma	.47→ 43.9	3.4(1.5)	-46.4%	169.3→ 224.6 - No major complications
41	Trauma	4.8→ 74.0	7.1(1.1)	-45%	102.0 - No major complications
42	Burn	4.7→ 31.0	1.8(.4)	-87.6%	70.2→ 1.0→ .3→ 5.2→ 75.6→ 76.5 - Major complications

Patient	Injury	PGE_2 [a]	$TNF\alpha$ [b]	PHA [c]	% T Cell Response [d] and Outcome [e]
43	Burn	7.5 → 19.0	6.98(0)	-87.1%	71.9→ 102.0→ 127→ 49.3→ 111→ 6.3→ 31.0→ 43.8→ 62.5→ 81.9→ 110→ 92.1 - Major complications
44	Trauma	16.04 → 52.87	1.51(0.4)	-22%	82.1→ 141.7→ 63.6→ 96.0→ 112.9 - No major complications
45	Burn	9.65 → 14.13	4.1(0)	-77.5%	34.3→ 76.0→ 53.1→ 21.9→ 7.0 - MOF - Expired
46	Trauma	5.11 → 29.73	0.82(0.4)	-51.2%	68.5→ 76.9→ 115.2→ 124.2 - No major complications
47	Trauma	- - -	2.1(1.0)	- - -	139.6→ 164.6→ 157.8 - No major complications
48	Trauma	- - -	2.73(1.02)	-69.6%	5.6→ 2.1→ 42.4 - No major complications
49	Burn	- - -	- - -	-4.5% *	No major complications
50	Trauma	.71→ 7.3	4.3(0)	-74%	6→ .2→ 2.6→ 1→ 15.4 - MOF - Expired
51	Trauma	4.2→ 7.3	.47(1.1)	+15%	No major complications
52	Burn	.4→ 17.7	9.6(0)	-55%	68→ 17→ .6→ 8→ 9→ 5.5→ 1.3→ .2→ 1.5→ 10→ 4→ 64→ 39→ 133→ 106 - MOF - Recovered
53	Trauma	.92	1.5(0)	-45%	76→ 99 - No major complications
54	Trauma	.72→ 38.4	4.2(.6)	-95%	.1→ 11→ 1→ 2→ .4→ 206→ 47→ 134→ 93- MOF
55	Trauma	6.4	.68(.78)	-72% *	75 - No major complications
56	Burn	.4→ 3.4	2.6(0)	-85%	9→ 83→ 12→ 7→ 13→ 5 - Major complications
57	Trauma	- - -	0.7(0.8)	-85%	61 - No major complications
58	Burn	- - -	2.56(0)	-96%	8.3→ 75.2→ 10.5→ 6.0→ 11.3→ 4.5→ 69.9→ 81.2 - Major complications
59	Trauma	- - -	1.57(0)	-91%	1.2→ 0.5 - MOF - Expired
60	Burn	- - -	0.24(0)	-89%	104.8→ 43.4→ 171.1→ 49.4→ 28.9 - No major complications
61	Burn	- - -	0.45(0.2)	-63%	69→ 73→ 49→ 45→ 127 - No major complications
62	Burn	- - -	0.54(0.2)	+75%	67→ 72→ 57→ 109→ 70→ 175→ 102→ 68 - Major complications
63	Burn	- - -	1.7(0)	-98%	105→ 1→ 0.6→ 0.5→ 0.5→ 85→ 70→ 39→ 0.6→ 0.5→ 84→ 10 - MOF - Expired
64	Trauma	- - -	0.98(0.77)	-98%	48→ 4→ 23→ 44→ 85→ 100→ 94 - Major complications
65	Trauma	- - -	0.37(0.77)	-57%	18→ 23→ 77→ 38→ 85 - Major complications
66	Trauma	- - -	0.77(0)	-69%	7 - No complications
67	Trauma	- - -	1.2(0.77)	-78%	118→ 4→ 2→ 16→ 2→ 18 - Major complications
68	Trauma	- - -	0.5(0)	-70%	81→ 28 - No complications

a. Maximal change in MØ prostaglandin E_2 (PGE_2) levels post-injury. PGE_2 is measured in a sensitive ELISA assay system and expressed as ng/10^6 MØ/ml.

b. Maximal levels of tumor necrosis factor (TNF_α) (secreted plus cell-associated) production by unstimulated patient (normal) MØ. TNF_α is measured in the L-M cell bioassay and expressed as ng/10^6 MØ/ml.

c. Maximal percent change of the patients' peripheral blood mononuclear cells(PBMC)proliferation response to 2μg/ml phytohemagglutinin mitogen compared over time to baseline value.
* Calculated as compared to paired normal since there was no baseline value for the patient.

d. Patient SRBC rosette purified T cell response to anti-CD3 (1μg/ml) and anti-CD4 (1μg/ml) on different post-injury days expressed as percent median of normal values simultaneously run.

e. Minor complications were excluded and defined as a single episode and single source of infection without systemic sequelae or hemodynamic instability. Major complications included multiple sources of infections, repeated episodes of infection, and multiple organ failure.

Table II - Tumor Necrosis Factor-Alpha (TNFα) Levels by Trauma Patients' Monocytes

Groups and Conditions	TNFα Levels (ng/10^8 MØ/mL)[a] released	membrane-associated	total
Normal Controls			
unstim[b]	0.0 (0.0- 6.6)	0.0 (0.0- 6.5)	0.0 (0.0- 11.5)
+MDP[c]	0.0 (0.0- 0.5)	0.0 (0.0- 7.2)	0.2 (0.0- 18.1)
+MDP +IFNγ[d]	4.8 (0.0- 167.5)	1.2 (0.0- 11.0)	7.4 (0.0- 173.3)
Phase II Patients			
unstim	0.0 (0.0- 10.3)	1.9 (0.0- 234.8)***	2.7 (0.0- 234.8)***
+MDP	0.4 (0.0- 31.3)*	3.6 (0.0- 169.3)***	5.6 (0.0- 169.3)***
+MDP +IFNγ	8.8 (0.0- 259.1)*	4.6 (0.0- 371.1)***	19.2 (0.0- 374.5)***
Phase III Patients			
unstim	0.0 (0.0- 0.2)†	16.9 (0.0- 578.2)***,††††	16.9 (0.0- 578.2)***,††††
+MDP	0.0 (0.0- 1.9)**,†††	18.3 (0.0- 503.4)***,††††	18.4 (0.0- 503.9)***,††
+MDP +IFNγ	0.4 (0.0- 73.0)**,††††	9.7 (0.0- 450.6)***,†	16.6 (0.0- 450.6)**

Table III - Comparison of MØ IL-10 [a] Levels and p75-TNFR [b] Shedding in Trauma Patients

	Normal [c]		Phase II [d]		Phase III [e]	
Stimulation	**TNFR**	**IL-10**	**TNFR**	**IL-10**	**TNFR**	**IL-10**
Unstimulated	2.3(0.2-6.3)	0.6(0.0-4.8)	1.7(0.0-8.8)	0.1(0.0-1.7) *	0.8(0.0-6.5) **	0.04(0.0-0.6) **
+MDP [f]	4.4(0.4-12.9)	2.2(0.0-12.0)	3.3(0.2-16.9)	0.5(0.0-7.3) **	1.9(0.3-8.1) *	0.09(0.0-1.1) **

* $p \leq 0.001$ [g] ** $p \leq 0.0001$

a. IL-10 measured in a sensitive ELISA expressed as ng/10^6 MØ/mL

b. p75-TNFR measured in a sensitive ELISA expressed as ng/10^6 MØ/mL

c. Median (range) of 49 normal individuals run simultaneously with patients.

d. Median (range) of 27 patients.

e. Median (range) of 22 patients.

f. 20μg/mL final concentration.

g. Statistical significance was determined by the two-sided Mann-Whitney U Rank Test.

Table IV - IL-10 Downregulates MØ TNFα Production in Normals and Patients

Experiment	MØ TNFα [a] (ng/10^6MØ/ml)			
	Normal		Patient [b]	
	MDP [c]	MDP+IL-10 [d]	MDP	MDP+IL-10
1	5.65	<0.05	17.64	12.17
2	5.65	<0.05	20.2	5.4
3	2.37	<0.05	7.53	4.34
4	1.34	<0.05	35.6	4.23
5	12.36	6.25	22.57	16.90
6	1.26	<0.05	26.0	<0.05
7	3.97	0.78	8.33	1.05
8	4.82	1.43	11.13	2.86
9	2.97	<0.05	5.54	<0.05
	P Value [e] =	0.0076	P Value =	0.0077

a. TNF_α levels, cell associated (MØ lysate) plus secreted (MØ supernates), were assessed by LM bioassays.

b. 9 samples were collected from 4 patients at different post-injury days.

c. MØ ($3x10^6$ cells/ml) were cultured in medium and MDP (20μg/ml) for 16 hours.

d. MØ ($3x10^6$ cells/3mls) were cultured in medium, MDP (20μg/ml) and IL-10 (50U/ml) for 16 hours.

e. Statistical significance (P) between MDP vs MDP+IL-10 stimulated TNF_α values was determined by Wilcoxon non-parametric test.

Table V

Anergy correlates to loss of T lymphokine production, MODS, and monocyte TNFα deregulation

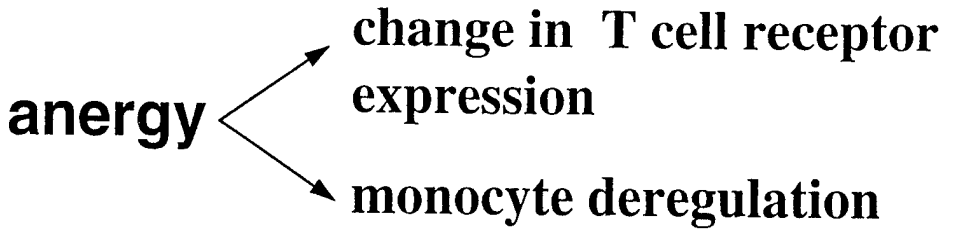
	mean % NL T cell prolif.	mean MODS score	Mean % of normals' T cell cytokine levels			
			IL-2 (Th1)	IFNγ (Th1)	IL-4 (Th2)	IL-10 (Th2)
anergic Phase III	**13.6%**	**7.6**	**2.5%**	**30.5%**	**11.8%**	**16.9%**
nonanergic Phase II	**105.7%**	**4.0**	**83%**	**230.6%**	**110.5%**	**307.9%**

Monocyte mTNF/TNFR ratio > 100:

anergic 80%

nonanergic 0%

Table VI



	MODS	% NL IL-2	TNF ratio >100
anergic	7.6	2.5	80%
nonanergic	4.0	83%	0%

Table VII

Anergy correlates to loss of apoptosis and depressed lymphokine production

	mean % of normals':		
T cell proliferation	T cell proliferation	IL-2	% apoptosis compared to NL
anergic (>50% depressed)	**13.6%**	**2.5%**	**0.2% below normals**
nonanergic (<50% depressed)	**105.7%**	**83%**	**4.2% above normals**

Table VIII Depressed responsiveness of trauma patients' anergic T cells to IL-12

	IFNγ (IU/10^6 cells/ml)[a]					
	Normal[b]			Phase III Patients[c]		
	Ab[d]	Ab+IL-12	Ab+IL-2	Ab	Ab+IL-12	Ab+IL-2
Median	23	87	37	1.0	2.7	4
Range	5-116	26-285	7.5 - 154	0.1 - 6	(0.7 - 19)	(0.5 - 35)

a. SRBC- rosette purified T cells isolated from normal controls and trauma patients were cultured ($2x10^5$ cells/200µl/well) in the presence of immobilized anti-CD3 (1 µg/well) + anti-CD4 (1µg/well) and the supernates were harvested after 24 hrs and assessed for IFNγ by ELISA.
b. Normal control sample (n=13) was run along with each patient's sample.
c. Patients' phase III of T cell responses (n=13) was determined on the basis of their T cell proliferation in the PBMC versus their isolated T cell population.
d. Ab= Immobilized anti- CD3(1µg/well) + anti -CD4 (1µg/well).

Table IX Simultaneous depression of proliferation and IL-2 production of trauma patients' anergic T cells[a] in response to Ab or PMA

	Proliferation (DPMx10^{-3})				IL-2 (pg/10^6cells/ml)			
	Ab		PMA		Ab		PMA	
	Nor[b]	Pt[c]	Nor	Pt	Nor	Pt	Nor	Pt
Median	143	13	41	3	1036	28	50	25
Range	106-236	6-15	15-69	0.1-9	659-2784	19-68	28-514	16-38

a. SRBC-rosette purified T cells from normal controls (n=6) and trauma patients (n=6) were simultaneously induced with anti-CD3+ anti-CD4 (Ab) or PMA for the assessment of proliferation (72 hrs. of culture) by [^{3}H] thymidine incorporation and IL-2 production (24 hrs. of culture) by ELISA.
b. Normal
c. Patient

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Appendix A

Only a Subset of Trauma Patients with Depressed Mitogen Responses Have True T Cell Dysfunctions

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Many studies have demonstrated depressed mitogenic responses in trauma/burn patients' peripheral blood mononuclear cells (PBMC). However, data attributing the relative contribution of secreted inhibitory factors versus a true T cell dysfunction to these depressed mitogenic responses have been conflicting. We have characterized the T cell dysfunctions in posttrauma mitogen depression by simultaneously assessing patient T cell proliferation in the phytohemagglutinin-stimulated PBMC and in the purified T cell population induced with anti-CD3 + anti-CD4. Patients' samples showed three distinct patterns or progressive phases of T cell responses: (i) normal or elevated T cell proliferation in both the whole PBMC and the isolated T cell population (phase I); (ii) depressed T cell proliferation in the PBMC but normal, or even elevated, proliferation in the isolated T cell population (phase II); and (iii) depressed T cell proliferation in both the PBMC and the isolated T cell population (phase III). Patients whose T cells exhibited only a phase I response experienced no major complications with a positive clinical outcome. Patients whose T cell alterations progressed to phase II experienced infectious episodes and some complications, but all had positive clinical outcomes. In contrast, patients whose T cells progressed to phase III dysfunction had severe clinical complications (multiple organ failure), with a negative clinical outcome (80% mortality). Patients whose T cells had a phase I or phase II response pattern had no true T cell dysfunctions in the absence of monocytes. However, patients whose T cells had a true T cell dysfunction (phase III) response pattern were at high risk for mortality. Thus, a true T cell dysfunction, though occurring in only a minority of trauma patients, is predictive of clinical outcome.

INTRODUCTION

Immunosuppression following burn and traumatic injuries has been shown to predispose patients to a high incidence of sepsis and related multiple organ failure (1–4). Impairment of T lymphocyte functional activity, as reflected by depressed proliferative responses to mitogen and/or reduced IL-2 producing capacity, is particularly implicated in the posttrauma immune suppression seen in both humans and murine models (1–7). A majority of the studies assessing the T cell mitogen responsiveness following hemorrhage, trauma, and/or burn have been carried out using either whole peripheral blood mononuclear cells (PBMC) or murine splenocytes (1–7). There have also been numerous reports of posttrauma excessive monocyte (MØ) production of inhibitory factors, such as prostaglandin E_2 (PGE_2) and transforming growth factor-β (TGFβ), compromising immune function (7–11). Thus, excessive inhibitory MØ activity can also cause depressed mitogen responsiveness postinjury. Depressed antigen presenting capacity of monocytes/macrophages has also been demonstrated following hemorrhage and trauma and also contributes to depressed mitogen responsiveness posttrauma (12, 13). Induction of suppressor T cells in thermal and nonthermal mechanical injury has been reported in different animal models, and such T cells appear to have a negative immunomodulatory effect on cell-mediated immunity (as reviewed in 14). A shift from Th1 to Th2 cells has recently been demonstrated posthemorrhage and during the course of polymicrobial sepsis in murine models (15, 16). Thus, the overproduction of Th2-type cytokines, such as IL-4 and IL-10, which are inhibitory to antigen presenting cell (APC) induction of Th1 cells, could also be responsible for posttrauma depressed mitogen responsiveness. However, the relative contributions of monocyte-secreted inhibitory factors, depressed APC (MØ/B cells) function, and/or independent true T cell dysfunction to the depressed mitogen-induced proliferation posttrauma and their relationship to clinical outcome remain to be elucidated. Recently, Ertel *et al.* have reported that the proliferation of purified T cells remains unaltered on all days posttrauma compared to healthy controls (17). In this report, we have simultaneously assessed the proliferative responses of T cells from patients with mechanical or thermal trauma in two different systems: (i) proliferation of T cells in the presence of APC (in the whole PBMC population) in response to mito-

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TABLE 1

Correlation of Maximal T Cell Alteration with Clinical Parameters

Maximal T cell alteration	M/F	Mean age (years)	Mean ISS[a]	Mean % burn	% Major complications[b]	% Mortality
Phase[c] I		45	25	31	0	0
n = 10 (7T[d] + 3B[e])	7/3	(29–85)	(17–41)	(12–50)		
Phase II		44	35	36	20	0
n = 20 (10T + 10B)	15/5	(20–77)	(19–57)	(6–86)		
Phase III		55	37	49	100	80
n = 10 (4T + 6B)	8/2	(28–74)	(24–57)	(15–80)		

[a] ISS, injury severity score reported for mechanical trauma patients.

[b] Minor complications were excluded and defined as a single episode and single source of infection without systemic sequelae or hemodynamic instability. Major complications included multiple sources of infections, repeated episodes of infection, and organ failure.

[c] Patients' phase of altered T cell function was determined on the basis of their T cell proliferation in the PBMC versus their isolated T cell population.

[d] Trauma patients.

[e] Burn patients.

gen–phytohemagglutinin (PHA) (APC influenced) and (ii) proliferation of T cells in the purified T cell population, i.e., in the absence of APC, in response to immobilized anti-CD3 + anti-CD4. This anti-CD3/CD4-induced T cell proliferation, although less vigorous than proliferation in the PBMC system, is APC independent. The patients' T cell data were analyzed and the T cell proliferative responses in the APC-influenced versus APC-independent systems were correlated to their clinical outcome.

MATERIALS AND METHODS

Patient Population

A total of 40 patients admitted to the University of Massachusetts Medical Center Trauma Unit (Worcester, MA) were included in this study. There were 21 patients with mechanical trauma (injury severity score >17) and 19 patients with thermal trauma (>25% total-body-surface burn or lower, if adjusted for advanced age). Their ages ranged from 20 to 85; the median age was 44. There were 30 men and 10 women. Ninety-eight samples from 40 patients were assessed at different postinjury days. Table 1 illustrates the severity of injury and outcome of the patients in this study. Patient samples were collected one to two times per week until the day the patient was released or expired. Many of the patients included in our study received anesthesia and/or blood transfusion on admission. Because of the known transient immunosuppression in the peripheral blood cells caused by anesthesia and blood transfusion which has no correlation with the ultimate clinical outcome, data from the samples drawn either on Day 0 (admission day) or Day 1 were not included in this study. Normal controls were tested along with each patient's samples. The control samples were collected from 34 normal donors consisting of 14 men and 20 women. Their ages ranged from 18 to 60; the median age was 40. Volunteers from laboratory and hospital staff at the University of Massachusetts Medical Center served as normal controls. Informed consent was obtained from all patients and controls and the study was approved by the Institutional Review Board.

Separation of PBMC

PBMC from normals' or patients' blood were isolated by centrifugation of heparinized blood over a Ficoll–Hypaque gradient at 1600 rpm for 20 min. The cells were washed two times with HBSS and then suspended in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with 8% Mishell Dutton tested serum (Life Technologies, Inc., Grand Island, NY), 50 units/ml penicillin G, 50 μg/ml streptomycin, 50 μg/ml gentamycin, 2.5 μg/ml fungizone, 4 mM L-glutamine, 1 mM Na pyruvate, and 1% minimal essential medium nonessential amino acids (Irvine Scientific). Endotoxin contamination was less than 15 pg/ml in the culture medium and serum, and all the media contained 100 U/ml polymyxin B sulfate (Calbiochem Corp., La Jolla, CA). A fraction of the PBMC were PHA stimulated as below and the rest used as the source of purified T cells and stimulated as below.

Proliferation of T Cells in the PBMC Population

PBMC (2×10^5 cells/200 μl/well) were cultured in flat-bottomed microtiter plates (Becton Dickinson, Lincoln Park, NJ) in the presence or absence of 1 μg/ml of PHA (Murex Diagnostics Ltd, Dartford, England). Cells were cultured for 72 hr and the cell cultures were pulsed with 1.0 μCi of [^{3}H]thymidine/well 18 hr before harvesting. Samples were counted in a beta counter.

The results are presented as mean disintegration per minutes (dpm) of triplicate cultures. When the proliferation of patients' PBMC was at least <30% compared to paired normal value, it was considered depressed.

Purification of T Cells and Their Proliferation

T cells were purified from PBMC as previously described (18, 19). Briefly, normals' and patients' PBMC were depleted of MØ by selective adherence to a microexudate-treated plastic surface. Nonadherent cells were rosetted with neuraminidase (Sigma Chemical Co, St. Louis, MO)-treated sheep red blood cells (SRBC). The SRBC-rosetted cells were essentially free of monocytes, as determined by flow cytometric analysis. These cells were >90% T cells with <1% contamination by B cells and monocytes. The purified T cells were suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 containing 10% FBS (Sigma) and all other supplements, as mentioned above, for the culture of PBMC except the Mishell Dutton tested serum. The cells were cultured (2×10^5 cells/200 μl/well) in flat-bottomed microtiter plates in the presence of immobilized anti-CD3 and anti-CD4. Proliferation of T cells in response to anti-CD3 is not dependent on the presence of APC (e.g., monocyte) (20). mAb were immobilized onto plastic microtiter plates as described (18, 20). In brief, anti-CD3 (AMAC, Inc., Westbrook, ME) diluted in RPMI 1640 was placed (1 μg/50 μl/well) in each of the wells of 96-well flat-bottomed microtiter plates (Becton–Dickinson), incubated at room temperature for 1.5 hr, and then washed twice with PBS to remove nonadherent mAb. The process was repeated with anti-CD4 (Biosource International, Camarillo, CA) (1 μg/50 μl/well). T cells were cultured for 72 hr and, 18 hr before harvesting, the cell cultures were pulsed with 1.0 μCi of [^{3}H]thymidine/well. Samples were counted in a beta counter and the results are expressed as mean dpm of triplicate cultures. When the proliferation of patients' purified T cells was at least <30% compared to paired normal value, it was considered depressed.

Generation and Assessment of Soluble Factors

PBMC (2×10^5 cells/200 μl/well) and SRBC rosette-purified T cells (2×10^5 cells/200 μl/well) were cultured in a 96-well microtiter plate for 24 hr in the presence of PHA (1 μg/ml) or immobilized anti-CD3 (1 μg/well) + anti-CD4 (1 μg/well), respectively. Culture supernates were harvested and stored at −80°C until the day of assay for PGE_2 or any specific cytokine. Assessment of PGE_2 in the culture supernates of PBMC was done by ELISA kit (Amersham International plc, England) according to the instructions of the manufacturer. The sensitivity of the assay was 16 pg/ml. IL-10 and IFN-γ levels in the culture supernates of PBMC and isolated T cells were assessed by specific ELISA (IL-10 kit, Biosource International; and IFN-γ kit, Medgenix Diagnostics SA, Belgium) according to the instructions of the manufacturers. The sensitivity of the IL-10 ELISA kit was 5 pg/ml and that of the IFN-γ ELISA kit was 0.03 IU/ml.

Statistical Analysis

Because there exists a wide individual variation in human T cell proliferation and cytokine levels and the data obtained in our experiments are not normally distributed, parametric statistical analysis (i.e., mean and standard deviation) is inappropriate. Although a normal control sample was run along with each patient sample, the data are not paired in statistical analysis. Hence, the Mann–Whitney nonparametric U test (Macintosh Statview) was performed for the calculation of the level of significance (P) between normal and patient values.

RESULTS

Three Different Patterns (Phases) of T Cell Proliferative Responses

When 98 PBMC samples collected from 40 trauma/burn patients at different postinjury days were assessed for T cell proliferation in response to PHA, 34 samples showed normal or even elevated proliferation, while 64 samples exhibited depressed mitogenic responses. Similar data for the majority of burn/trauma patients have been reported by several groups (1–3, 5, 9). To analyze the relative contribution of an inherent T cell dysfunction to this depressed PBMC mitogenic response, T cells were simultaneously purified from all the patients' PBMC samples. These APC free T cells were then assessed for T cell proliferation in response to immobilized anti-CD3 + anti-CD4 along with the PHA-induced PBMC T cell cultures. T cell proliferation induction to anti-CD3 + anti-CD4 does not require APC (20). Purified T cell proliferation was then compared with the PBMC T cell proliferation. When the T cell proliferation data in the isolated (purified) and mixed (PBMC) populations were analyzed carefully, three distinct T cell response patterns were observed: (i) normal or elevated T cell proliferation in both the PBMC (PHA induced) and the isolated T cell (anti-CD3 + anti-CD4 induced) population (Fig. 1), (ii) depressed PHA-induced proliferation of PBMC but normal or even elevated isolated T cell proliferation in the concomitantly assayed response to anti-CD3 + anti-CD4 (Fig. 2), and (iii) depressed T cell proliferation in both the PBMC (PHA induced) and the isolated T cell population (anti-CD3 + anti-CD4 induced) (Fig. 3). To assess our data more simply and to reflect any progressive relationship of the three T cell response patterns, we have introduced the term "phase" for these three different T cell

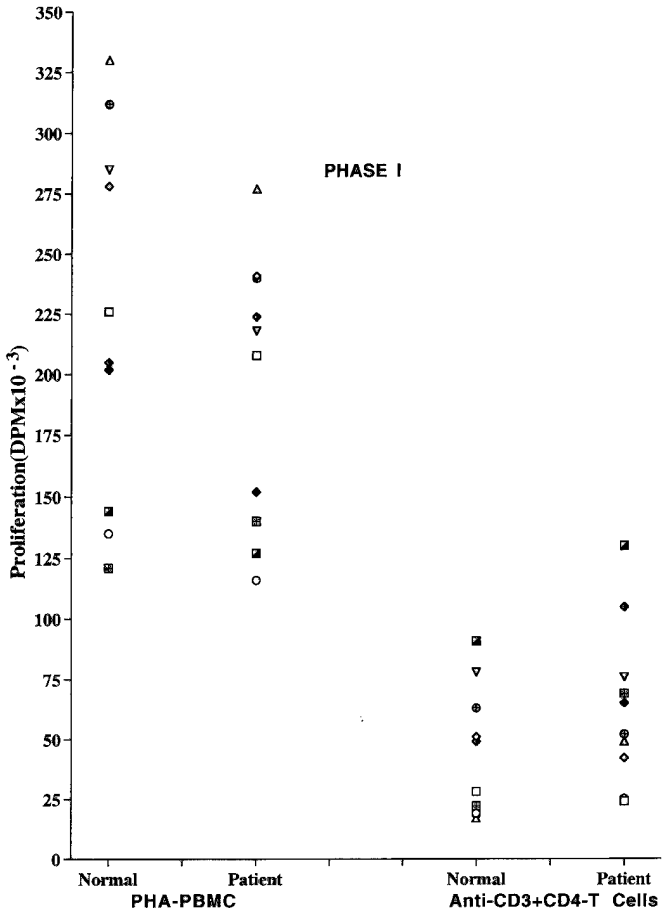

FIG. 1. Normal or elevated T cell proliferation both in the mixed (PBMC) and in the isolated T cell populations. The figure depicts the data of 10 representative samples covering the entire range from a total of 34 samples collected from 21 patients on different postinjury days. A normal control sample was run along with each patient's sample. PBMC and purified T cells (2×10^5 cells/200 μl/well) were assessed for proliferation ([^{3}H]thymidine uptake) in response to PHA (1 μg/ml) and immobilized anti-CD3 (1 μg/well) + anti-CD4 (1 μg/well), respectively, for 72 hr of culture and the results are expressed as dpm. Statistical significance (P) between normal and patient values was tested on all 34 samples by the Mann–Whitney nonparametric test.

response "patterns." T cells from patients exhibiting the first type of T cell response pattern are designated phase I response, while T cells exhibiting the second and third response patterns are designated phase II and phase III responses, respectively.

The phase I response exhibits no depressed T cell proliferation, either in the PBMC or in the isolated T cell population. The phase II T cell response pattern represented depressed T cell responses when the T cells were assessed with a mitogen-induced PBMC population but not when the T cells were isolated and simultaneously assessed in the anti-CD3 + anti-CD4 system. Thus, the patients with phase II T cell response pattern have no true T cell depression. Consequently, phase II type depression of PBMC T cell proliferation may be due to the presence of inhibitory PBMC-derived factors and/or the presence of aberrant APC cell types (i.e., MØ, B cells) other than T cells. When the levels of PGE_2 (a MØ-secreted T cell inhibitory molecule) in the supernates of PBMC from patients with phase II T cell responses were assessed, PGE_2 levels were found to be significantly ($P = 0.0001$) higher than those of normals' PBMC (Table 2). Thus, the increased PGE_2 levels may be one of the contributors to the phase II type depressed T cell responses in the patients' PBMC population.

The phase III response pattern of depressed T cell

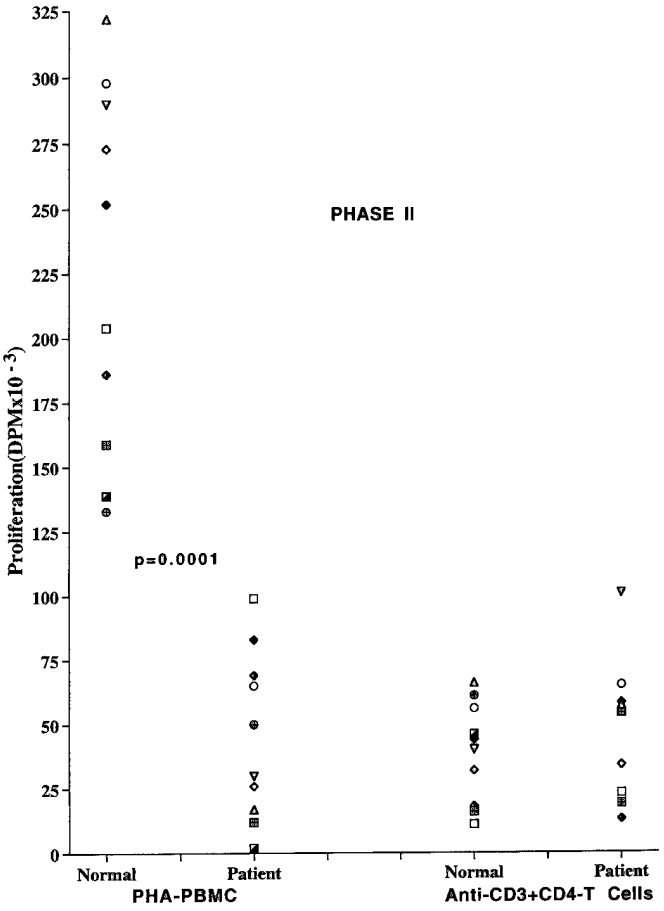

FIG. 2. T cell proliferation is depressed in the mixed (PBMC) population but normal or even elevated in the isolated T cell population. The figure depicts the data of 10 representative samples covering the entire range from a total of 44 samples collected from 24 patients at different postinjury days. A normal control sample was run along with each patient's sample. PBMC and purified T cells (2×10^5 cells/200 μl/well) were assessed for proliferation ([^{3}H]thymidine uptake) in response to PHA (1 μg/ml) and immobilized anti-CD3 (1 μg/well) + anti-CD4 (1 μg/well), respectively, for 72 hr of culture and the results are expressed as dpm. When the proliferation of PBMC or purified T cells is at least 30% less than that of the paired normal, it is considered depressed. Statistical significance (P) between normal and patient values was tested for all 44 samples by the Mann–Whitney nonparametric test.

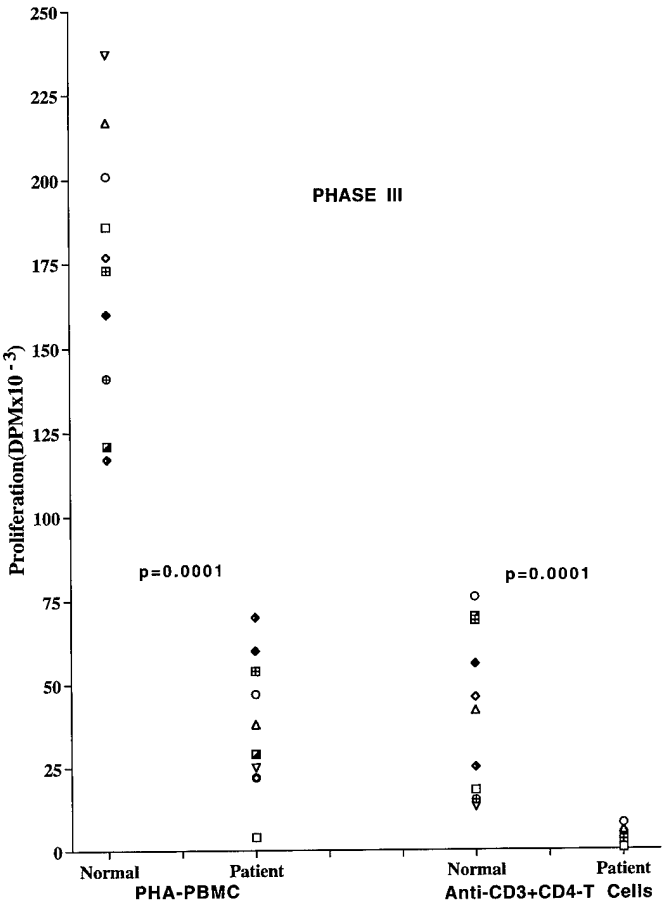

FIG. 3. Depressed T cell proliferation in both the mixed (PBMC) and the isolated T cell population. The figure depicts the data of 10 representative samples covering the entire range from a total of 20 samples collected from 10 patients at different postinjury days. A normal control sample was run along with each patient's sample. PBMC and purified T cells (2×10^5 cells/200 μl/well) were assessed for proliferation ([^{3}H]thymidine uptake) in response to PHA (1 μg/ml) and immobilized anti-CD3 (1 μg/well) + anti-CD4 (1 μg/well), respectively, for 72 hr of culture and the results are expressed as dpm. When the proliferation of PBMC or purified T cells is at least 30% less than that of the paired normal, it is considered depressed. Statistical significance (P) between normal and patient values was tested for all 20 samples by the Mann–Whitney nonparametric test.

proliferation, which occurred even in the isolated T cell population, suggested the presence of a T cell dysfunction independent of any MØ or other PBMC-secreted inhibitory factors. When the PGE_2 levels in the supernates of PBMC from patients with phase III T cell response pattern were assessed, PGE_2 levels were found to be depressed rather than elevated (Table 2). Thus, the MØ-secreted factor, PGE_2, does not seem to be the immediate cause of phase III type of depressed T cell responses either in the PBMC or in the isolated T cell population. Recently, a shift from Th1 → Th2 cells has been implicated as occurring after hemorrhage in a murine model, suggesting that the trauma patients having depressed T cell responses in PBMC (phase II and phase III response patterns) should be assayed for a shift from Th1 (e.g., IFN-γ) to Th2 (e.g., IL-10) type of cytokine production. As can be seen from Table 3, patients experiencing phase II and phase III response patterns of T cell depression exhibited simultaneously depressed IFN-γ and IL-10 levels in their PBMC population. However, in phase II, where isolated T cell proliferation was elevated or normal, both IFN-γ and IL-10 levels in the isolated T cell culture supernates were also simultaneously elevated. In contrast, patients' isolated T cells exhibiting a phase III type T cell response had depressed anti-CD3/anti-CD4-induced proliferation concomitant with depressed levels of IFN-γ and IL-10 in their culture supernates. Consequently, a shift from Th1 to Th2 cells, as characterized by decreased IFN-γ and increased IL-10 levels, does not seem to be the immediate cause for either the phase II or phase III type of depressed T cell responses in these patients. However, in phase I, the isolated T cells had normal levels of IFN-γ and IL-10, whereas their PBMC had normal IFN-γ but depressed IL-10 levels. The depressed PBMC IL-10 levels appear to result from depressed MØ IL-10 levels, rather than any depression in T cell IL-10 production (18). Thus, our data indicate that in phase I, patient T cells function almost normally. In phase II, patient isolated T cells are normal to hyperactive, indicating no true T cell dysfunctions, despite their depressed PBMC PHA responses. However, in phase III, T cells are unresponsive, with some dysfunctions independent of APC.

Transition of Phases Posttrauma

Figure 4 gives the summary of the percentages of T cell proliferation patterns/phases of all the patients'

TABLE 2

PGE_2 Levels Are Elevated in Phase II but Depressed in Phase III in PBMC from Trauma Patients

	PGE_2 (pg/10^6 cells/ml)[a]		
	Normal[b]	Phase II (patient)[c]	Phase III (patient)
Median	5400	16300	2660
Range	2040–14500	7000–36800	128–8150
Sample size	29	17	12
p value[d]		0.0001	0.006

[a] PBMC were cultured (2×10^5 cells/200 μl/well) in the presence of PHA (1 μg/ml) and the supernates were harvested after 24 hr and assessed for PGE_2 by ELISA.

[b] Normal control sample was run along with each patient sample.

[c] Patients' phase of altered T cell function was determined on the basis of their T cell proliferation in the PBMC versus their isolated T cell population.

[d] Statistical significance (p) between normal and patient values was determined by Mann–Whitney U test.

TABLE 3

Simultaneous Measurement of IFN-γ and IL-10 Levels in the Supernates of PBMC and Isolated T Cells

Median IFN γ[a] (IU/10^6 cells/ml) (range)				Median IL-10[b] (pg/10^6 cells/ml)			
	Patient[d]				Patient		
Normal[c]	Phase I	Phase II	Phase III	Normal	Phase I	Phase II	Phase III
PHA—PBMC[e]							
27.2 (11–106)	29.0 (7–85)	8.5*[,f] (0.5–31)	1.4* (<0.05–14)	4695 (1127–11230)	1457** (339–3683)	790** (146–4234)	273* (<0.05–737)
Anti-CD3+CD4—T cells[g]							
45.9 (9–121)	37.0 (10–158)	96.4*** (22–245)	7.7* (<0.05–17)	117 (46–722)	146 (53–2045)	270*** (101–896)	20.5* (<0.05–72)

[a] IFN-γ levels in the culture supernates were assayed by ELISA.
[b] IL-10 levels in the culture supernates were assayed by ELISA.
[c] Normal control sample (n = 33) was run along with each patient sample.
[d] The patients' phase of altered T cell function (n = 10 for phase I, n = 13 for phase II, n = 10 for phase III of trauma patients) was determined on the basis of their T cell proliferation in the PBMC versus their isolated T cell population.
[e] PBMC (2×10^5 cells/200 μl/well) were cultured for 24 hr in the presence of PHA (1 μg/ml), and the supernates were harvested for the assessment of IFN-γ and IL-10.
[f] Statistical significance (P) between normal and patient values was assessed by Mann–Whitney U test: *P = 0.0001, **P = 0.0002, and ***P = 0.002.
[g] MØ-depleted and SRBC rosette-purified T cells were cultured for 24 hr in the presence of immobilized anti-CD3 (1 μg/well) + anti-CD4 (1 μg/well), and the supernates were harvested for the assessment of IFN-γ and IL-10.

samples tested. Although there were three different T cell response phases among the trauma patient samples, one patient could experience phase I, phase II, and phase III types of T cell responses over his or her postinjury clinical course. Figure 5 shows the transition of T cell responses from phase I to phase II in a patient over time postinjury. Transition from phase II to phase III type of T cell response in another patient

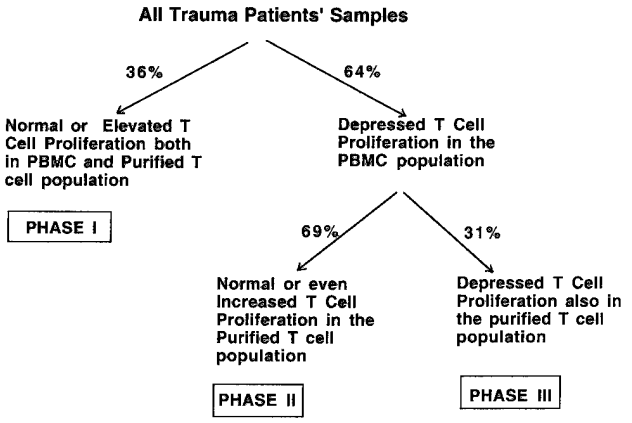

FIG. 4. Summary of the T cell proliferative (both in the PBMC and in the isolated T cell population) response pattern/phase of all the 98 samples collected from 40 patients at different postinjury days. PBMC and purified T cells (2×10^5 cells/200 μl/well) were assessed for proliferation ([^{3}H]thymidine uptake) in response to PHA (1 μg/ml) and immobilized anti-CD3 (1 μg/well) + anti-CD4 (1 μg/well), respectively, for 72 hr of culture. When the proliferation of PBMC or purified T cells is at least 30% less than that of the paired normal, it is considered depressed.

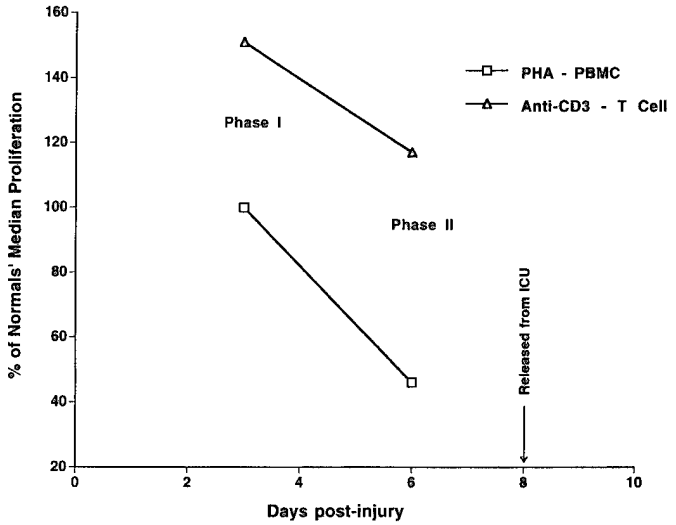

FIG. 5. Transition from phase I to phase II in a patient over time postinjury. Proliferation of PBMC in response to PHA (PHA–PBMC) and of T cells in response to anti-CD3 + anti-CD4 (anti-CD3-T cell) at any postinjury day are expressed as the percentage of the median of normal proliferation values.

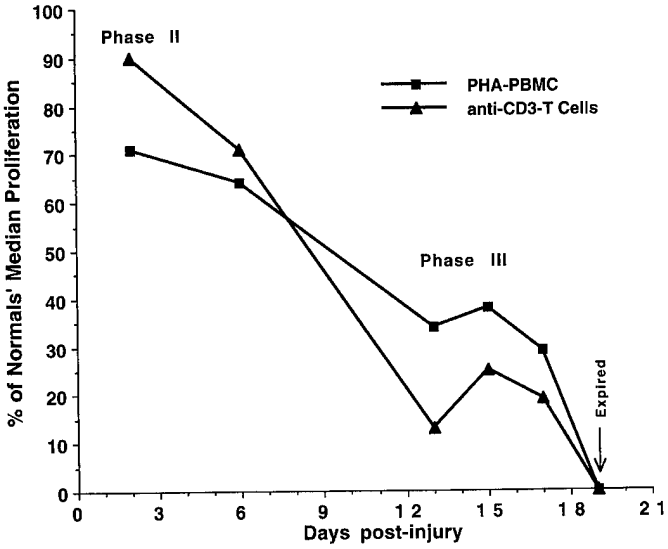

FIG. 6. Transition from phase II to phase III in a patient over time postinjury. Proliferation of PBMC in response to PHA (PHA–PBMC) and that of T cells in response to anti-CD3 + and anti-CD4 (anti-CD3-T cell) at any postinjury day are expressed as the percentage of the median of normal proliferation values.

is shown in Fig. 6. Figure 7 illustrates a transition of T cell responses from phase II to phase I. Figure 8 depicts the transition between the phases of T cell responses over time postinjury in a patient who was released from the ICU after 60 days. Our overall data suggest that the phase of the T cell response might be progressive, i.e., starting from phase I and progressing to phase II, or even further to phase III, although a transition from phase I directly to phase III was observed in a few of the patients studied. It is possible that we had missed phase I in a few patients whose T cell responses started from phase II. However, because the blood samples could not be collected from the same patient every day over his or her postinjury time course (not permitted by the Institutional Review Board), a transient phase of T cell response alteration might have been missed. Such a missed observation could explain the direct transition of T cell responses from phase I to phase III or starting the T cell response pattern from phase II in those few patients.

Association of Phase of T Cell Response Pattern to Clinical Outcome

The T cell response pattern or phase of the trauma patients appears to correlate with clinical outcome. Table 4 gives the summary of the progression of patients' altered T cell response phases to their final outcome. Trauma patients who experienced only phase I T cell responses over their entire postinjury period predominantly had uneventful clinical courses and these patients were quickly released from the ICU. Patients whose T cells progressed to a phase II type of dysfunction (but did not progress to phase III) had moderate infectious episodes but positive outcome. All of these patients survived without major clinical complications. However, the patients whose T cell dysfunction progressed to phase III (true T cell dysfunction) had major clinical complications. Eight of 10 patients who experienced a phase III T cell response pattern had multiple organ failure and finally died; 2 other patients survived after prolonged hospitalization and repeated septic episodes (45 and 60 days) in the ICU. Figure 8 exhibits the data of 1 of the 2 surviving patients who experienced a phase III type of T cell response at 7 days postinjury. This patient's T cell response returned to a phase II pattern and he was finally released from the ICU after 60 days. This patient had multiple clinical complications throughout his hospital stay. As can be seen in Table 1, the median age of the trauma patients whose T cell response pattern progressed to phase III (55 years) was slightly higher than the median age of the patients who experienced only phase I (45 years) or even progressed to phase II (44 years), which may suggest that age may be a contributing factor for the progression of the T cell dysfunction. But the T cell response of three patients of ages 28, 35, and 39 years also progressed to phase III, causing multiple clinical complications and a mortality rate of 2 of 3. Similarly, other patients 71, 72, 77, and 85 years old never progressed beyond phase I or phase II. Thus, the age of the patient does not seem to be the major contributing

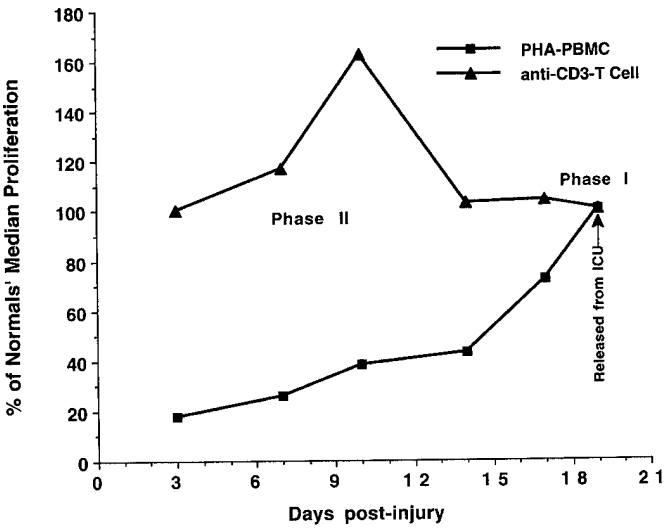

FIG. 7. Transition from phase II to phase I in a patient over time postinjury. Proliferation of PBMC in response to PHA (PHA–PBMC) and that of T cells in response to anti-CD3 + anti-CD4 (anti-CD3-T cell) at any postinjury day are expressed as the percentage of the median of normal proliferation values.

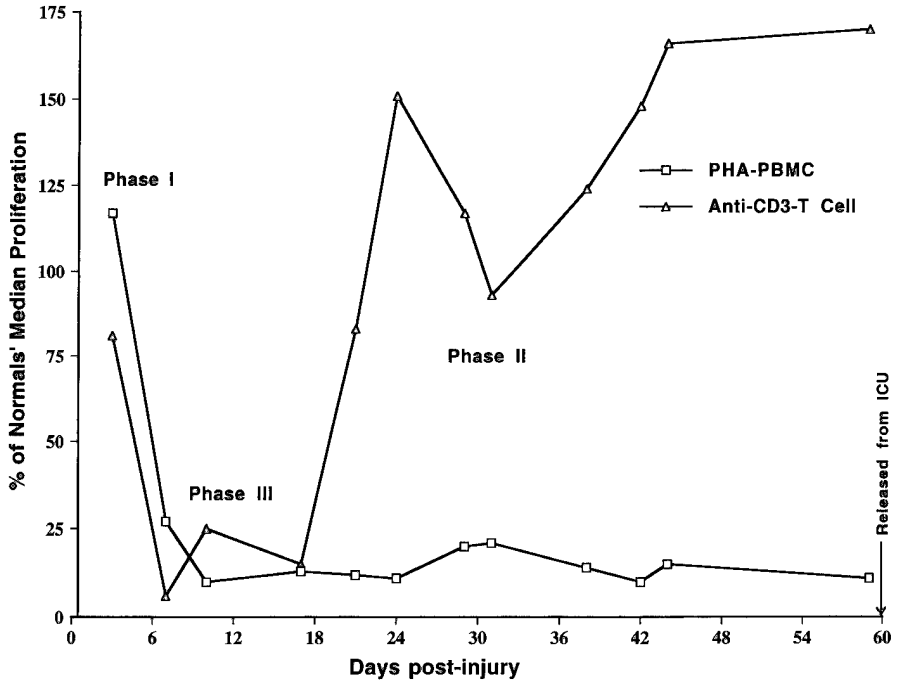

FIG. 8. Transition between the phases in a patient over time postinjury. Proliferation of PBMC in response to PHA (PHA–PBMC) and that of T cells in response to anti-CD3 + anti-CD4 (anti-CD3-T cell) at any postinjury day are expressed as the percentage of the median of normal proliferation values.

factor in the progressive dysfunction of T cells in the trauma patients.

DISCUSSION

The results presented here clearly indicate that a depressed T cell proliferation in response to PHA in the trauma patients' whole PBMC population does not always indicate a true T cell dysfunction in these patients. Although T cells proliferate in response to PHA, there is also a requirement for macrophage/monocytes as APC and a potential contribution of inhibitory factors from non-T cells. The majority of the patients' T cells we assessed exhibited a phase II response pattern.

TABLE 4

Association of the Trauma Patients' Phase[a] of Altered T Cell Function with Their Clinical Outcome

	No. of patients	Clinical outcome
Experienced only phase I	10	All the patients survived
Proceeded to phase II	20	All the patients survived
Proceeded to phase III	10	8 of 10 patients died

[a] Patients' phase of altered T cell function was determined on the basis of their T cell proliferation in the PBMC versus their isolated T cell population.

That is, their depressed T cell proliferation in response to PHA was due to some altered activity of other cells present in the PBMC population since their isolated T cells exhibited normal or even elevated proliferative responses in a direct stimulation system (immobilized anti-CD3 + anti-CD4). However, a small number of trauma patients' samples exhibited a phase III pattern of T cell responses showing proliferation depression both in their PBMC and in their isolated T cell population, indicating some true T cell dysfunctions. There are several possible causal mechanisms for the depressed T cell proliferation in response to PHA in phase II type of T cell responses. Increased MØ production of PGE_2 following trauma, burn, or hemorrhage has been well established in clinical and animal models (8, 9, 14). Increased PGE_2 levels posttrauma have been shown to be responsible for depressing the production of IL-2, an essential cytokine for the development of proliferative T cell responses to antigenic and mitogenic stimulus (7, 9, 10). In this report, when the PGE_2 levels in the culture supernates of PBMC from patients with phase II T cell responses were tested, they were found to be significantly elevated. Thus, increased PGE_2 levels could contribute some of the depressed mitogen responsiveness in these patients. In a few experiments, addition of indomethacin, a cyclo-oxygenase inhibitor, to our PBMC culture showing a phase II pattern of T cell depression partially restored the mitogen-induced

PBMC proliferation, further implicating PGE_2 as a suppressor factor (data not shown). MØ production of TGFβ has also been demonstrated to be elevated in trauma patients (11, 14). Increased TGFβ could also be responsible for the phase II type of depressed PBMC T cell responses. Defective MØ antigen presenting capacity following hemorrhage/trauma has been repeatedly reported and also can be one of the causal mechanisms for the phase II pattern of depressed PBMC T cell responses (12, 13). The possibility of the generation of suppressor T cells following mechanical or thermal trauma has been well documented (as reviewed in 14). In addition, contamination of very immature polymorphonuclear leukocytes in the Ficoll–Hypaque-separated PBMC population, resulting in a decrease in the relative number of T cells, has been reported in burn patients (21). We have observed similar T cell depletion even in our mechanical trauma patients (unpublished observation). Thus, the decrease in T cell numbers in the patient PBMC population can also account for some of the depressed PBMC T cell proliferation. However, in the experiments with the isolated T cell population, the patient T cell numbers were adjusted and equivalent to the normal control. Recently, a shift from Th1 to Th2 cells resulting in overproduction of IL-4 and IL-10 has been demonstrated posthemorrhage and during the course of polymicrobial sepsis in murine models (15, 16). The same phenomenon could be responsible for depressed PBMC T cell responses in the patients with phase II T cell responses. However, we have previously shown that depressed mitogenic responses of PBMC from trauma patients are not due to elevated levels of IL-10 (18). In fact, PBMC IL-10 levels were depressed posttrauma (18). Here, we have tested both Th1 (e.g., IFN-γ) and Th2 (e.g., IL-10) type cytokines and found them simultaneously depressed in the culture supernates of PBMC while simultaneously elevated in the purified T cells with a phase II response pattern. Th1 $\rightarrow$ Th2 shift does not, therefore, appear immediately causal for depressed PBMC mitogenic responses of patients with a phase II type T cell response pattern. However, we cannot dismiss the possible ultimate immunosuppressive effect of increased and persistent levels of Th2 type cytokines even in the face of simultaneous elevated IFN-γ in eventually generating unresponsiveness.

Normal levels of proliferation in the PHA-stimulated purified T cell populations of mechanical trauma patients on all the postinjury days have been reported by Ertel *et al.* (17). Thus, our data showing normal or elevated proliferation of the anti-CD3/anti-CD4-induced purified T cells in the majority of the patients' samples (phase I and phase II) are consistent with the findings of Ertel *et al.* (17).

The causal mechanism for depression of patients' T cells in phase III response pattern is unaddressed in these experiments. Patients' isolated phase III T cells did not proliferate in response to anti-CD3 + anti-CD4, reflecting some true dysfunctions in these T cells irrespective of the inhibitory role, if any, of other cell types (MØ, B cells) in the PBMC population. Similarly, a shift from Th1 to Th2 cells does not seem immediately causal of patients' phase III T cell depression, as both IFN-γ and IL-10 levels were simultaneously depressed in both PBMC and purified T cell supernates. However, phase III T cell unresponsiveness could result from induction of anergy. Such anergy induction might be a result of persisting aberrant levels of Th2 cytokines during the transition through phase II. Prolonged stimulation of normals' T cells in the absence of APC also leads to anergy (22). Depressed APC functions have been reported in trauma patients (13). Thus, the repeated infectious challenge posttrauma could chronically stimulate T cells in the absence of appropriate APC activity, eventually initiating T cell anergy. T cell anergy has been reported in severe burn patients (23). Alternatively, activation-induced T cell death, or apoptosis, might be occurring when the phase III T cells were stimulated *in vitro*. Increased apoptosis has been demonstrated in thymocytes following sepsis in murine models (24–26). Recently, Theodorczyk-Injeyan *et al.* found increased apoptotic death of T cells in patients with severe thermal trauma (27). Consequently, patients' phase III T cells might be undergoing severe apoptosis during *in vitro* induction, thus preventing proliferation in the 72-hr experimental period. Alternatively, there could be a possibility of differential distribution of $CD4^+$ and $CD8^+$ T cell subsets postinjury, resulting in altered T cell responses. However, use of anti-CD3 + anti-CD4 as stimulants still would reveal a T cell dysfunction. We observed that anti-CD3 alone, which induces both $CD8^+$ and $CD4^+$ T cells, could stimulate almost 80% of the total proliferation compared to that induced by the combination of anti-CD3 + anti-CD4 (data not shown). Our data cannot eliminate the possibility that the T cell dysfunction in phase III results from expansion of an unusual CD3 negative T cell subset.

Depressed proliferation in the purified patient T cell population observed in the phase III response pattern is inconsistent with the data reported by Ertel *et al.* (17). This apparent conflict may reflect differing trauma severity in the two patient populations. In our study, 8 of 10 patients exhibiting depressed proliferation in their purified T cell population died. In contrast, only 1 of 21 patients who showed normal proliferation in the purified T cell population died in the studies of Ertel *et al.* (17). In our studies, no patient with normal or elevated proliferation in the purified T cell population died. Thus, the two studies agree that normal T cell proliferation capacity portends a positive clinical outcome. Our study goes further to show that patients

with inherently depressed T cell proliferation capacity are at increased risk of mortality and that development of this T cell dysfunction occurs progressively over time postinjury in some but not most patients.

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Normal MØ Cell-Associated TNF_α Is Resistant to PGE_2 as Well as TGF_β Downregulation[a]

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Eighty percent of patients with severe thermal or mechanical trauma who die more than 2 days postinjury succumb not as a direct result of their injuries, but from organ failure (kidney, lung, and heart) due to cytokine and septic shock.[1] These immunoaberrant trauma patients experience both depressed T-lymphocyte function with subsequent increased septic episodes and elevated monocyte (MØ) tumor necrosis factor alpha (TNF_α) of both cell-associated and secreted types.[1,2] Normal human MØ TNF_α production is downregulated by both prostaglandin E_2 (PGE_2) and TGF_β at the mRNA level.[3,4] However, trauma patients with depressed T-cell proliferation responses (immunoaberrant) because of excessive MØ PGE_2 production also experience excessive TNF_α production concomitant to increased MØ TGF_β production.[5,6] This report compares the sensitivity of paired trauma patients' and normal control subjects' MØ TNF_α production to the addition of exogenous PGE_2 or TGF_β. Because trauma patients' MØ produce large excesses of biologically active cell-associated TNF_α (cell TNF_α), both cell-associated and secreted TNF_α were assessed.

Patients with severe mechanical (injury severity score >35) or thermal trauma (>30% 3° burn) and normal controls were selected for this study. Mitogen responses were assessed. Patients with depressed responses were classified as immunoaberrant and selected for further study of the monocyte (MØ) functions. Trauma patients' and normal subjects' MØ were separated by selective microexudate adherence of the Ficoll-Hypaque density isolated mononuclear cells.[2] In some experiments, normal MØ were cross-link stimulated through their 72-kD FcγRI by rosetting the T-cell-depleted mononuclear cells with anti- Rh-coated erythrocytes, followed by density isolation of the rosetted cells, then further purification by adherence to microexudate-coated plates.[2] MØ equaled 95% of the final population by CD14 staining. The isolated MØ population, FcγRI-stimulated and FcγRI-nonstimulated MØ (3×10^6 MØ in 3 ml of medium), was further induced with 20 μg/ ml of muramyl dipeptide (MDP) or a combination of 3 hours' priming with either 10 U or 100 U IFNγ/ml plus 20 μg/ml MDP. Then 10^{-7} *M* PGE_2 or 2.4 ng/ml TGF_β was added with the IFN-γ primer. Appropriate ethanol controls were included in the PGE_2 experiments. Using

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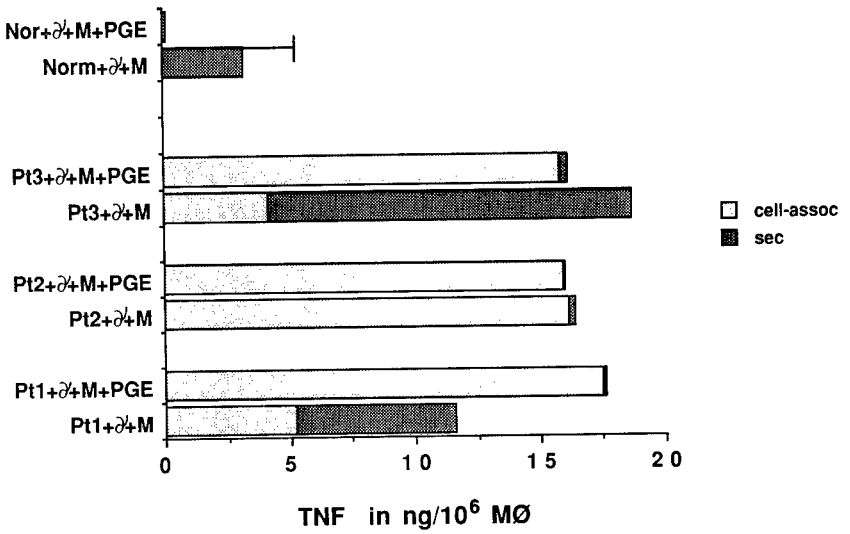

FIGURE 1. PGE_2 addition downregulates both patients' and normals' Secreted TNF_α. 10^6/ml trauma patient (Pt) or normal (Nor) MØ cultured with 10 U IFN-γ plus 20 μg/ml MDP (γ + M) in the presence or absence of 10^{-7} *M* exogenously added PGE_2. TNF_α was measured in LM bioassay as described. Cell-associated TNF_α (cell-assoc) was measured in MØ membrane sonicates, whereas secreted TNF_α (sec) was measured in MØ supernatants. Normal TNF_α values represent the medium and range for paired normals.

the LM bioassay, secreted TNF_α was measured in the MØ supernatants, and cell-associated TNF_α activity was determined in the sonicated MØ lysates after repeated freezing-thawing.[2] Both secreted and cell-associated MØ TNF_α activity was totally neutralized by anti-TNF_α antibody. PGE_2 was measured by ELISA in the MØ supernatants.[2] MØ supernatants were assayed for TGF_β using the mink lung bioassay against a recombinant human TGF_β standard, as described.[2]

Patients' MØ producing PGE_2 levels of >30 ng/10^6 MØ and TGF_β levels of 50-200 pM 10^6 MØ were concomitantly producing elevated total TNF_α levels (secreted and cell-associated) of 30 to 130 ng/10^6 MØ/ml. These data implied that ongoing patients' MØ TNF_α production is resistant to downregulation by both PGE_2 and TGF_β. However, new induction of MØ TNF_α might still be sensitive to PGE_2 or TGF_β downregulation. The exogenous addition of 10^{-7} *M*/ml PGE_2 to patients' or normal subjects' MØ stimulated with a suboptimal IFN-γ prime (10 U/ml) plus MDP resulted in suppression of new TNF_α production by both normal and immunocompetent trauma patients' MØ, while having no significant effect on total MØ TNF_α levels induced in immunoaberrant patients, but significantly altering the ratio of secreted to cell-associated TNF_α in the aberrant trauma patients' MØ (FIG. 1). Exogenous TGF_β (2.4 ng/ml) added to the same suboptimal stimulation system had no inhibitory effect on patients' MØ secreted or cell-associated TNF_α, whereas induction of normal MØ-secreted TNF_α was significantly inhibited (TABLE 1). Because of the differential inhibitory effect of PGE_2 on patients' secreted *versus* cell-associated TNF_α, we investi-

TABLE 1. TGF_β Increases MØ TNFα Levels in IFN-γ + MDP-Stimulated Trauma Patients' MØ While Decreasing TNF_α in Controls' MØ[a])

	TNF Bioactivity			
	Secreted/Cell-Associated (ng/10^6 MØ)			
	Nor	Pt	Nor	Pt
10 IFN-$_\gamma$ + MDP	15.8/1.6	23.1/4.5	4.0/0	10.2/24.7
10 IFN-$_\gamma$ + MDP + TGF_β	4.9/2.1	32.4/3.5	3.1/0	11.4/27.4
10 IFN-$_\gamma$ + MDP	9.5/5.7	38.1/7.3	11.4/2.6	8.7/24.3
10 IFN-$_\gamma$ + MDP + TGF_β	5.9/4.7	38.2/7.8	6.9/3.2	12.2/29.6
10 IFN-$_\gamma$ + MDP	15.1/0	38.1/10.3	2.1/0	10.5/0.3
10 IFN-$_\gamma$ + MDP + TGF_β	12.4/0	105.8/16.2	1.6/0	17.1/0.3

[a] TNF_α activity in LM bioassay of 3×10^6 normal control (Nor) or trauma patients' (Pt) MØ stimulated by 10 U/ml IFN-$_\gamma$ + 20 μg/ml MDP in the presence or absence of 2.4 ng/ml TGF_β. Secreted TNF_α was assayed in MØ supernatants and cell-associated TNF_α in MØ lysates.

gated the effect of the exogenous addition of either PGE_2 or TGF_β in a system in which normal MØ were induced to produce cell-associated as well as secreted TNF_α. Cross-linking the normal MØ FcγRI receptor induces significant MØ cell-associated as well as secreted TNF_α.[2,5] In this system, normal MØ cell-associated TNF_α induced by FcγRI cross-linking was resistant to PGE_2 downregulation and TGF_β inhibition. However, secreted TNF_α was still inhibited by the addition of either TGF_β or PGE_2.

These data suggest that although immunoaberrant patients' MØ are concomitantly producing high levels of TNF_α, TGF_β, and PGE_2, new induction of secreted TNF_α is still sensitive to PGE_2 inhibition, whereas cell-associated TNF_α induced before the addition of PGE_2 is resistant to inhibition in both patients and normal subjects. In contrast, TGF_β inhibits induction of normal subjects' MØ-secreted TNF_α but not patients' secreted TNF_α. The posttrauma induction of high levels of PGE_2 resistance to cell-associated TNF_α, along with the altered sensitivity of TNF_α to TGF_β inhibition can contribute to excessive TNF_α production by trauma patients' MØ.

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Appendix C
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Increased Monocyte Tumor Necrosis Factor-Alpha Message Stability Contributes to Trauma Patients' Increased TNF Production

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Running Title: Increased TNF Message Stability In Trauma Patients

Abstract

Post-trauma elevation of TNF_α appears critical in mediating many symptoms of Systemic Inflammatory Response Syndrome (SIRS) resulting in late mortality. Although increased MØ TNF_α production plays a pivotal role in this TNF_α elevation, the molecular mechanisms leading to increased MØ TNF_α production have yet to be elucidated. Here, we demonstrate that although TNF_α mRNA levels are increased in all trauma patients' MØ which produce elevated levels of TNF_α protein, in the majority of patients, these increased TNF_α mRNA levels are under normal transcriptional and post transcriptional control. Consequently, the increased TNF_α production by these patients' MØ is likely due to preactivation of these MØ by trauma released mediators. However, a small minority of patients whose mortality rate was 57%, produce TNF_α of primarily the membrane-associated type. The MØ TNF_α mRNA accumulation of these patients in response to *in vitro* stimulation is significantly augmented. All of these patients experienced SIRS. In this subset of patients' MØ, TNF_α mRNA stability was aberrantly increased. Such an increase in TNF_α mRNA stability could lead to devastatingly prolonged production of TNF_α protein. This demonstration of increased TNF_α mRNA stability in post trauma MØ represents a novel correlation of elevated membrane-associated TNF_α protein, increased mortality, and a mechanism for this occurrence.

Key Words: Systemic Inflammatory Response Syndrome, Monokines

Introduction

Systemic inflammatory response syndrome (SIRS)[1] is the most common pathologic sequela of burn and trauma. Excess cytokine production mediates many of the pathologic symptoms of SIRS such as multiple organ failure and adult respiratory distress syndrome (ARDS) (1-8). Increased TNF_α levels have been demonstrated as pivotal in mediating some of the symptoms of SIRS (5, 9-13). When TNF_α is produced in small amounts or in a localized fashion, it likely plays a beneficial role in acute injury or inflammation (14-16). However, a massive upregulation of TNF_α production has been associated with multiple organ failure and death (1-8, 10, 11, 17-23).

Monocytes (MØ) have been demonstrated to be major producers of the elevated TNF_α seen post trauma (4, 7, 24, 25). After trauma, MØ are exposed to injury generated mediators, such as complement split products and substance P, which have been shown to induce increased TNF_α production. We and others have data suggesting that a majority of the highly augmented TNF_α produced subsequent to trauma remains in the 27 kD cell-associated form (6, 23, 26, 27). Therefore, post trauma MØ TNF_α increases can remain undetected unless the MØ themselves are examined for cell-associated TNF_α.

The molecular basis of increased MØ production of TNF_α subsequent to trauma has yet to be completely elucidated. It is possible that no increases in TNF_α mRNA occur after trauma and that the TNF_α protein released is due to mediators present in the post trauma environment increasing the processing of the TNF_α protein from its precursor to its bioactive form. Alternatively, increased TNF_α protein output could result from the trauma induced release of preformed protein which has been stored in the cell until secondary signals induce its release. Finally, the post trauma increase in TNF_α protein production could be due to increased TNF_α message levels or increased translation of this mRNA. This could result from increased stimulation or from the deregulation of the tightly controlled translation or transcription of TNF_α mRNA (28-38)

Stimulated MØ TNF_α mRNA levels have been shown to be influenced by a number of factors (39-44). Variation in the transcription, translation, or stability of the message can cause changes in the amount of this mRNA in the MØ. In addition, a prior activation by one inducer can dramatically alter the mRNA production induced by a secondary stimulus. Specifically, measles virus has been

shown to depress the amount of TNF_α protein induced by a subsequent LPS stimulus by decreasing the stability of the TNF_α message (45). Conversely, Influenza A virus has been shown to increase the stability of this mRNA species thereby leading to an increase in the amount of TNF_α protein produced in response to a subsequent LPS stimulus (46). Consequently, stimulation of MØ by trauma induced mediators could also alter these MØ's TNF_α production in response to a subsequent bacterial challenge

In this investigation, MØ from 15 trauma patients and 22 burn patients who showed increased MØ TNF_α protein production were assessed for increased TNF_α mRNA levels. Detected increased TNF_α mRNA production was then further related to both types of TNF_α protein produced (cell-associated and secreted) and to possible changes in mRNA regulation to determine whether the increase in TNF_α protein production seen in trauma patients correlates with increased and/or deregulated TNF_α mRNA production. The low numbers of recoverable patient MØ coupled with the need for simultaneous analysis of patients' MØ for cell-associated and secreted TNF_α protein production, made direct measurement of RNA transcription using nuclear run on technology impractical. Even Northern blot quantitation of cellular TNF_α mRNA accumulations was often unfeasible due to low MØ numbers. Consequently, the mimic quantitative polymerase chain reaction system, a well established means of quantitating mRNA levels using small amounts of message, was used to assess patients' MØ TNF_α mRNA levels and quantitatively compare these levels to those of normals' MØ (47-51). Finally, the stability of the TNF_α message in patients' MØ was evaluated in actinomycin D time course experiments.

In this study, it was determined that all patients' MØ which produced increased levels of TNF_α protein also had increased levels of TNF_α mRNA accumulation. However, a small subset of patients with increased mortality and/or major clinical complications appear to have MØ whose TNF_α mRNA exhibited abnormally increased stability. These patients were also producing primarily $mTNF_\alpha$ rather than secreted TNF_α.

Methods

Patient Population. Patients were selected from those admitted to the University of Massachusetts Medical Center Trauma Unit or Burn Unit, Worcester. Trauma patients (10 male, 5 female)with severity scores >13 and burn patients (18 male, 4 female) with > 5% total body surface 2-3^{o} burns, depending upon age, were included in this study. The average age of patients was 49 years (range 25-85 years). Sample collection was conducted on a semi-weekly basis beginning upon admittance to the hospital and ending upon release from the intensive care unit. The sample collection normally constituted a course of less than three weeks during which any one patient was involved in the study. Normal controls' MØ samples were assayed concomitantly with each patient's sample. Normal controls consisted of 25 volunteers from laboratory and hospital staff at the University of Massachusetts Medical Center (ages 18-58 years; average, 40 years). Informed consent was obtained from all patients and controls. The protocol for sample collection was reviewed and approved by the University of Massachusetts Human Subjects Review Committee.

Monocyte Separation and Stimulation. Monocytes from patients' and normals' blood were isolated from Ficoll-Hypaque gradient-separated mononuclear cells (PBMC) by selective adherence as described previously (52). Briefly, nonadherent cells were removed after 1.5 hours culture on microexudate-coated plastic surfaces. Adherent cells were then removed from the surface using 10 mM EDTA and were re-plated at $1x10^{6}$ cells/ml in RPMI 1640 medium (JRH Biosciences, Lenexa, KS) supplemented with 15% FBS (Sigma Chemical Co., St. Louis, MO), 50 U/ml penicillin-G, 50 ug/ml streptomycin, 50 ug/ml gentamycin, 2.5 ug/ml fungizone, 4mM L-glutamine, 1mM sodium puruvate, and 1% MEM nonessential amino acids (JRH Biosciences, Lenexa, KS) on tissue culture plates (Corning Inc., Corning, NY). This procedure yielded >95% pure MØ.

Endotoxin contamination was less than 12 pg/ml in the culture media and FBS. All cultures contained polymixin B to prevent any carried over LPS from activating the patient MØ. Patient and normal MØ were then cultured in media only (unstimulated) or were stimulated with 20 ug/ml muramyl dipeptide (MDP; generously provided by CIBA-GEIGY, Basel, Switzerland).

TNF_α Bioassay. TNF_α activity in MØ supernates (secreted TNF_α) and in sonicated MØ lysates (cell-associated TNF_α) were measured in the LM cell (derivative of L929, a murine connective tissue cell line) bioassay as previously described (53). Both cell-associated and secreted MØ TNF_α activity were totally inhibited by anti-TNF_α neutralizing antibody.

Statistical analysis. Because there exists a wide individual variation in human T cell proliferation and cytokine levels and the data obtained in our experiments are not normally distributed, parametric statistical analysis (i.e. mean and standard deviation) is inappropriate. Although a normal control sample was run along with each patient sample, the data are not paired in statistical analysis. Hence, the Mann-Whitney nonparametric U test (Macintosh Statview) was performed for the calculation of the level of significance (p) between normal and patient values.

Molecular Studies. Isolated MØ were either induced with MDP or simply cultured for either two or sixteen to twenty hours. In the actinomycin D experiments, the cells were incubated as above for 2, 6, or 16 hours. In the actinomycin D experiments, 1 ug/ml actinomycin D was added to isolated MØ at two hours post induction. At indicated times, total cytoplasmic RNA was isolated using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH) according to manufacturer's instructions. Equivalent amounts of RNA were reverse transcribed using an oligo dT primer and M-MuLV reverse transcriptase (New England Biolabs, Beverly, MA). Reverse transcription reactions were incubated at 37°C for 3 hours to allow reactions to go to completion.

Resulting cDNA was used in competitive polymerase chain reactions (PCR) in which serial dilutions (2.5, 0.5, 0.1, and 0.02 attomoles / reaction) of TNF_α MIMIC (Clontech Laboratories, Palo Alto, CA) were used as competitors for cell derived TNF_α cDNA. Amplifications were carried out using human TNF_α amplimers (Clontech Laboratories, Palo Alto, CA) Competitive PCR was also performed using the Clontech G_3PDH MIMIC as competitor to facilitate standardization of samples. Human G_3PDH amplimers (Clontech Laboratories, Palo Alto, CA) were used in these amplifications. PCR was carried out in 50 ul using AmpliTaq DNA polymerase along with the included reaction buffer (Perkin Elmer, Foster City, CA).

Amplifications were carried out on a Perkin Elmer 480 Thermal Cycler. One cycle at 94°C for two minutes followed by three cycles of 94°C for one minute, then one minute at 56°C , and two minutes at 72°C were performed. This was followed by twenty seven cycles of one minute at 94°C, one minute at 60°C, and two minutes at 72°C followed by one cycle at 72°C for ten minutes. This amplification protocol was performed on all samples. PCR products were electrophoresed on 1.2% agarose TAE gels containing 0.1 ug/ml ethidium bromide. Bands were visualized and photographed on a Mighty Brite U.V. trans-illuminator (Hoeffer Scientific, San Francisco, CA). The intensity of bands was determined through computer based video densitometric analyses of gel photograph negatives using NIH Image software. Quantitation of original TNF_{α} RNA was determined by comparison of target and MIMIC band intensities. The results were standardized by G_3PDH quantities. Concentration curves were generated for each sample (Fig 1). Figure one is a procedural example of preliminary calculations for mimic quantitative PCR. All patient and normal MØ samples were amplified along with mimic amounts of 2.5 - 0.02 attomoles. This range covered the normal and patient TNFα mRNA concentrations encountered in the samples generated in these experiments.

Results

As we and others have previously described, most of the induced patients' monocytes (MØ) show increased TNF_α bioactive protein subsequent to trauma when compared to similarly induced normals' MØ (Table 1) (5, 6, 12, 13, 25, 26, 54). The TNF_α protein production by stimulated patients' MØ is much greater than the production of this protein by similarly stimulated normal MØ. However, normal MØ do respond to MDP stimulation with increased TNF_α protein production (data not shown). MØ were induced with the gram positive bacterial cell wall analog, muramyl dipeptide (MDP) rather than LPS in order to avoid the complication of post trauma modulation of CD14 (LPS receptor) from the monocyte surface. Loss of CD14 receptor expression has been described as occurring in activated MØ and could result in falsely low LPS responses (55). MDP induced MØ TNF_α protein production is normally seen as an increase, almost exclusively, in secreted protein (Table 1). However, the observed MØ TNF_α protein increase in a subset of trauma patients was seen almost exclusively as an increase in the amount of the cell-associated ($mTNF_\alpha$) protein measured as sonicated membrane bioactivity. This predominance of $mTNF_\alpha$ was most typical of patients with exaggerated TNF_α levels. In the patients with the greatest MDP induced TNF_α production increase, the amount of secreted TNF_α that was produced made up less than 1% of the total. Such a predominance of MØ cell-associated TNF_α after injury has been reported by several groups (6, 25-27) and is indicative of some post-trauma alteration in MØ TNF_α production. The apparent increase in TNF_α protein production by trauma patient MØ could be due to a true up-regulation of mRNA levels in these monocytes. Alternatively, these TNF_α protein increases could simply reflect increased efficiency of processing of the 32kD precursor TNF_α to its bioactive form or increased release of pre formed TNF_α protein.

In order to more clearly define any molecular basis for the increased TNF_α bioactivity in trauma patients' monocytes and to determine if these increased levels were due to a true protein production up-regulation, specific mRNA levels were examined using quantitative mimic PCR with mimic concentrations over a range of 2.5 - 0.02 attomoles as described in the methods section (Fig. 2A). Mimic quantitative PCR is a well established method for quantitation of low amounts of

RNA (47-51). All mRNA values were corrected for dilutions and were normalized according to G_3PDH levels in each sample. Subsequent to two hours' culture, matrix isolated normals' monocytes induced with 1ug/ml MDP accumulated a median of 78.0 attomoles of TNF_α specific mRNA per 10^6 cells (range 10.2-118.0 attomoles/10^6 cells). Similarly treated patients' MØ accumulated a median of 256.0 attomoles of TNF_α message per 10^6 cells (range 2.1-1020.0 attomoles/10^6 cells). Thus the increased amount of TNF_α bioactivity produced when assessing all patients' MØ is reflected in an increase in the amount of TNF_α mRNA accumulation in the cells.

An increased TNF_α mRNA production in MDP stimulated patients' monocytes is not surprising. The patients, as a result of their injuries, have likely been exposed to numerous *in vivo* MØ stimuli such as complement split products, substance P, etc., thereby priming their MØ for increased TNF_α production. As a result of these *in vivo* stimulations, patients' MØ should exhibit increased levels of TNF_α mRNA as compared to normals' MØ even without *in vitro* MDP stimulation. We found that normal MØ matrix adhered and cultured for two hours, without addition of MDP, accumulated a median of 56.0 attomoles of TNF_α specific mRNA per 10^6 cells (range 6.0-68.0 attomoles/10^6 cells) (Fig. 2B). In contrast, similarly matrix adherence stimulated patient monocytes accumulated a median of 78.0 attomoles of mRNA per 10^6 cells (range 25.0-230.0 attomoles/10^6 cells). These data combined with the data in figure 3A indicate that the trauma patients' MØ are *in vivo* activated and respond to both a minimal matrix adherence activation or an MDP stimuli with increased levels of TNF_α protein and TNF_α mRNA as compared to normals' MØ.

If the increased TNF_α mRNA levels produced by patients' MØ were due solely to their previous *in vivo* stimulation by trauma generated factors, subsequent *in vivo* stimulation should either result in minimal additional augmentation of transcription or, at most, a second increase of approximately equivalent magnitude to that seen in normal MØ. However, if the mRNA regulation in stimulated patients' monocytes is altered, an aberrantly large magnitude of TNF_α mRNA augmentation may be produced upon a second stimuli contributing to subsequent exaggerated TNF_α production and potentially to the development of SIRS. We simulate such a secondary bacterial challenge *in vitro* by culturing patients' and normals' monocytes with MDP. A comparison of MØ cultured with and without addition of MDP for two hours was conducted to allow quantitation of the

MØ's reaction to this treatment (Fig. 3). MDP treated normals' monocytes produced an average increase in TNF_α mRNA of 96% over their untreated levels. In contrast, MDP treated patients' monocytes increased production of their TNF_α message by an average of 351%. The increase in overall accumulation of patient MØ TNF_α mRNA subsequent to MDP stimulation followed two patterns. In the first pattern, seen in MØ of the majority of patients, MDP treatment resulted in elevations of TNF_α mRNA over untreated levels. These increases were similar in magnitude to the increases induced in normals' MØ although the quantities of the TNF_α mRNA of these untreated patients' MØ were initially higher than those of untreated normals. In a minority of trauma patients, however, a second pattern emerged. In these patients' monocytes, *in vitro* MDP addition lead to a much greater magnitude of TNF_α mRNA accumulation than was seen upon stimulation of normal monocytes. When considered as a group, monocytes from this small patient subgroup had message accumulations averaging 599% over their unstimulated levels.

Trauma patient monocytes produce increased levels of TNF_α protein and this increase is reflected by an increased accumulation of TNF_α message rather than release of preformed TNF_α. In the majority of patients (≈75%), this increase in TNF_α mRNA seems to reflect an *in vivo* pre-stimulation of patients' MØ since *in vitro* stimulation with MDP leads to an increase over untreated levels that is similar in magnitude to that seen in normals. However, a small number of patients (≈25%) had monocytes which responded to MDP treatment by an exaggerated accumulation of TNF_α mRNA possibly indicating some trauma induced aberration in regulation of TNF_α mRNA production.

An increase in mRNA stability is one of the mechanisms which has been shown to result in hyper-accumulation of TNF_α mRNA in stimulated monocytes (36, 56-59). In an effort to determine if TNF_α mRNA was aberrantly regulated in trauma patients, the persistence of this mRNA species in patient monocytes was examined. The amount of TNF_α mRNA persistent at a late time period (incubated with MDP for 16-20 hrs) is a function of the mRNA stability, the transcription rate, the promoter activation and other factors. These experiments were conducted to determine whether aberrant regulation of TNF_α mRNA was occurring. TNF_α mRNA levels in monocytes cultured with or without MDP were assessed using the quantitative PCR system at 2 hours and at 16 to 20 hours post MDP addition (Fig. 4). Using Mimic-PCR, the expression of TNF_α mRNA was clearly

detectable in the 2 hour samples from both normals' and patients' MØ. In the 20 hour samples, however, the expression of TNF_α mRNA was easily quantitatible in the patients' MØ samples but this mRNA species was barely measurable in the normals' MØ samples. This difference indicates a very low remaining or residual amount of the TNF_α mRNA species in the normal monocytes at 20 hours post induction. This low residual TNF_α mRNA level reflects the normal dynamic of a rapid increase followed by a rapid decrease of this message in MØ following stimulation (Fig. 4A). The G_3PDH housekeeping gene levels which were used to normalize the TNF_α levels, remained relatively constant throughout the experiment (Fig. 4B). As expected, all patient monocytes that had quantitatively greater levels of mRNA at 2 hours also had higher levels of TNF_α message remaining at the later time point. However, when the actual 16 or 20 hour residual mRNA level was calculated as a percentage of the initial 2 hour content, the patients' MØ again seemed to fall into two groups (Tables 2 and 3). In the first group, which were the majority of the tested patient samples, patients' monocytes had higher total amounts of mRNA but the percent residual levels were comparable to normal MØ (Table 2). The rapid decay of TNF_α message and other downregulatory mechanisms, such as transcription inhibitors which are pivotal in TNF_α regulation, appeared essentially normal in these low residual patients (60, 61). In the other group, patient monocytes appeared to retain abnormally high levels of the message as indicated by increased percent residuals (Table 3). These patients with abnormal high residuals were also the patients with increased $mTNF_\alpha$ (Table 5). Normal MØ cultured for 16-20 hours without MDP had an average residual TNF_α mRNA content equaling 7% of their 2 hour level. Similarly, the low residual group of cultured patient MØ cells had an average residual message content equaling 8% despite their initial higher TNF_α levels. Addition of MDP to the cultures led to a 5% average normal MØ residual message content after 16-20 hours' culture while MDP treatment of this group of patients' MØ led to an average residual message content of 4%. Therefore, the majority of trauma patients' monocytes elevated TNF_α mRNA levels appeared to result from their *in vivo* pre-activation. The residual TNF_α mRNA content of the majority of patients' MØ was, therefore, not significantly different from normals' MØ ($p=0.8955$ and $p=0.7676$ for uninduced and MDP induced residuals respectively) (Table 2). However, the second (high residual) patient group's average 16-20 hour residual MØ TNF_α mRNA level was 19.6% compared

to the simultaneously run normals' MØ average level of 9%. Culture with MDP led to a 3.9% average residual message level in the normal MØ while MDP treatment of this subgroup of patients' MØ led to an average residual message content of 21.9% (Table 3).

The increased residual TNF_α levels in both the uninduced and the MDP induced MØ from the high residual group of trauma patients were significant (p=0.0163 and 0.0039 respectively). These patients also produced elevated levels of cell-associated TNF_α protein upon stimulation with MDP and had negative clinical outcomes (Table 5). A variety of mechanisms could result in increased mRNA accumulation. Increased mRNA residual levels could be the result of a lack of normal transcriptional down-regulation of TNF_α. This lack of down-regulation could be due to altered induction of transcriptional inhibitors which are induced concomitantly with TNF_α message as has been described in other MØ stimulation systems (60-62). Alternatively, these data could be explained by increased TNF_α mRNA stability in these patients' monocytes. Increasing TNF_α mRNA stability has been shown to increase levels of both TNF_α mRNA and TNF_α protein (46).

Investigation of many of the possible mRNA regulatory pathways was not possible due to the limited numbers of cells available for this study. However, increased mRNA stability is a pivotal mechanism in altering MØ TNF_α mRNA levels and this mechanism could be examined. To determine the comparative stability of the message in the subset of patients with increased residual TNF_α mRNA, actinomycin D was added beginning at 2 hours post MDP induction to block transcription. Monocytes were harvested at 2, 6, and 16 hours post MDP stimulation and persisting TNF_α mRNA levels were assessed (Fig. 5). Again the TNF_α PCR products are clearly detectable in both normals' and patients' MØ samples at the 2 hour time point. However, at the 6 hour time point, the TNF_α mRNA has decreased in the normals' MØ samples yet are still predominant in the patients' MØ samples. Densitometric measurements confirmed the quantitative difference between patient and normal MØ TNF_α mRNA levels. Simultaneous Mimic-PCR analysis of the MØ G_3PDH expression affirmed that the amount of this species of mRNA varied little between normals' and patients' samples throughout the experiment. Densitometric assessment of the comparison G_3PDH mRNA levels were always used in normalizing the values reported for TNF_α mRNA. By 16 hours post stimulation, all but one treatment of one patient TNF_α mRNA residual percentage levels were higher than that of the

simultaneously assayed normal monocytes (Table 4). After MDP induction, the actinomycin D treated patient monocyte TNF_α mRNA levels persisted at an average of 24 x the level seen in the concomitantly assessed actinomycin D treated normal monocytes indicating increased TNF_α mRNA stability.

Discussion

Trauma patients' monocytes are exposed to many injury generated mediators which induce TNF_α production and could prime the MØ for greater production of TNF_α upon subsequent bacterial stimuli (36, 54). TNF_α protein production was dramatically increased in trauma patients' MØ induced *in vitro* with the bacterial cell wall analog MDP. In the 25% of patients whose TNF_α protein increased over three fold, the cell-associated form of TNF_α accounted for the vast majority of their increased production as has been reported by several groups (6, 23, 26, 27).

It has been postulated that the *in vivo* secondary stimuli which lead to increased TNF_α production may be repeated septic episodes which commonly follow trauma (2-4, 64). However, some investigators have noted no increase in TNF_α production by trauma patient monocytes subsequent to a secondary *in vitro* stimulation with LPS leading them to conclude that post injury TNF_α production is not increased (65, 66). This lack of an *in vitro* response to LPS is likely due to several factors. One of these factors is the fact that the post-trauma MØ TNF_α that is produced as the cell-associated form would not be detected in serum or cell supernate measurements. Another factor is a post trauma MØ unresponsiveness to LPS due to modulation of their CD14 LPS receptors from the cell surface a phenomenon which has been shown to occur in activated monocytes (50). To circumvent this, we have stimulated the MØ with the gram positive cell wall analog muramyl dipeptide (MDP) which utilizes a receptor different from CD14. This MDP stimulation induces a greater TNF_α response than does concomitant LPS induction in patients' MØ (data not shown).

A pre-activation of patient MØ was confirmed by data showing increased levels of patient TNF_α mRNA even without a secondary MDP stimulation. Such trauma mediator induced pre activation could account for all of the increased TNF_α protein seen when comparing trauma patients' MØ to normals' MØ. In fact, the increased TNF_α protein and mRNA in most patients' monocytes seemed to be the result of their pre-stimulation. Subsequent to MDP stimulation, the majority of

patients' monocytes responded with an average percent increase in TNF_α message accumulation and in TNF_α protein production which was equivalent to normal. However, there was a small group of patients whose monocytes' average increase in TNF_α message accumulation subsequent to MDP treatment was significantly greater than that seen in normals. This increased accumulation correlated with an increase in the TNF_α mRNA levels at 16 or 20 hours post induction. This increase was seen even when these mRNA values were normalized as a residual percentage of the corresponding 2 hour levels. This high residual mRNA content correlated with the expression of elevated levels of TNF_α protein of the cell-associated type. These elevated residual TNF_α mRNA levels appeared to result primarily from increased TNF_α mRNA stability.

This conclusion is supported by experiments in which non-MDP induced, Actinomycin D treated patient MØ had an average almost two fold higher residual TNF_α mRNA levels at 16 hours than did the normals. MDP induction prior to Actinomycin D treatment in these experiments led to patients' MØ possessing 22 fold higher TNF_α mRNA residual levels at 16 hours than did the induced normals' MØ.

Interestingly, in these Actinomycin D time course experiments, all of the normal 6 hour samples had lower TNF_α message levels than did the two hour samples. In the patient samples, however, 67% of the non Actinomycin D treated 6 hour samples showed accumulations of message that were at least slightly higher than the two hour accumulations. By 16 hours post stimulation, 67% of patient residual percentage levels were at least double that of the concomitantly assayed normals'. These data are consistent with a breakdown of TNF_α mRNA in normal individuals' MØ, but a delay in mRNA decay in this subset of patients' MØ.

Two mechanisms, pre activation and increased mRNA stability, seem to lead to increased TNF_α protein levels in trauma patients' monocytes. Pre activation of the MØ leads to increased expression of TNF_α protein (37,38) and primes the MØ for further increases in protein production subsequent to secondary stimuli. These TNF_α production increases appear to be under normal mRNA regulatory control and result in a MØ TNF_α response which is normal except for the increased amounts of protein produced. This pre activation apparently does not lead to any adverse clinical outcome. An increased production of TNF_α protein when under appropriate regulatory

control may even increase host resistance due to the availability of controlled pulses of increased levels of TNF_α.

In contrast, in a small proportion (≈25%) of the patients, increased stability of the $mTNF_\alpha$ mRNA led to dramatically increased levels of TNF_α protein upon secondary induction (Table 5). This increase is refractory to normal transcriptional control due to the fact that although new transcription can be halted, the existent, highly stable message will continue to be available in the cytoplasm for translation over an extended period of time. Therefore, in these patients, the possibly beneficial short pulses of TNF_α protein become prolonged waves of production of this cytotoxic cytokine whose clinical outcome could be devastating.

Several laboratories have investigated the expression of TNF_α. It has been determined that post transcriptional control plays a major role in this expression (41, 43). One study determined that, upon stimulation, MØ transcription of the TNF_α gene increased only three fold, TNF_α mRNA levels, on the other hand, increased nearly 100 fold, while TNF_α protein production increased 10,000 fold, dramatically demonstrating the pivotal role of post-transcriptional control in TNF_α production (41). Additionally, study of the 3' untranslated region (UTR) of the TNF_α gene has revealed the presence of a TTATTTAT consensus sequence which has been shown to have at least two regulatory roles. First, this sequence inhibits translation (67-69). Second, this sequence confers instability on the RNA, leading to a short half life (35).

Hayes, et al, in contrast, have shown that IFN_γ priming of MØ enhances LPS induced TNF_α protein production by enhancing the stability of TNF_α mRNA (37). Our data indicate increased stability of the TNF_α mRNA as a direct cause of increased expression of the TNF_α protein in some patients. However, these data cannot entirely rule out some accompanying loss of transcriptional regulation as a factor in the prolonged increase in TNF_α mRNA accumulation in these patients' monocytes.

The exaggerated increase in TNF_α mRNA in some of the patients' MØ is due to the fact that although the transcription of the RNA may be normal, this mRNA has increased stability which therefore exaggerates the magnitude of the accumulation. The most significant finding of this study was that 100% of the patients whose MØ produced TNF_α mRNA with increased stability experienced

major clinical complications and 57% went on to die. These patients' MØ were also producing primarily cell-associated rather than secreted TNF_α. Cell-associated TNF_α has been suggested as more cytotoxic to normal tissue and, therefore, may be more pathologic in SIRS (70). In contrast, only 7% of patients with normal MØ TNF_α mRNA stability experienced major complications and none of these patients died.

These data indicate that elevated monocyte TNF_α protein is expressed by a number of patients and is reflected in increased TNF_α mRNA levels. In the majority of these patients, this increase in MØ TNF_α protein and mRNA is apparently primarily due to *in vivo* pre activation of the MØ by trauma generated mediators. This increased MØ TNF_α activity may benefit patients by allowing a more rapid response to infections.

However, the significant results of these experiments, as summarized in Table 5, are that a small subgroup of patients develop altered TNF_α protein production indicated by an increased proportion of cell-associated TNF_α production, and increased mRNA accumulation and residual percentage, which is partially due to increased mRNA stability. More importantly, this aberration in TNF_α production correlated with increased patient morbidity.

Footnotes:

Abbreviations used:

SIRS - systemic inflammatory response syndrome

ARDS- adult respiratory distress syndrome

MØ - monocytes

MDP - muramyl dipeptide

cDNA- complementary DNA

G_3PDH - human glyceraldehyde 3-prostrate dehydrogenase

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Table1. Increased MØ TNFα bioactivity in trauma patients is detected primarily as cell associated form in some patients.

	Median TNFα (ng/10^6 MØ/ml)		
	Secreted[a]	**Cell Associated**[b]	**Total**[c]
Normal	**0.2**(0-13.8)	**0**(0-7.4)	**0.2**(0-13.8)
Group 1 Patients[d]	**4.8**(0-60.5)	**4.4**(0-13.8)	**9.2**(0-66.4)
Group 2 Patients	**0**(0-1.4)	**39.7**(8.8-167.1)	**39.7**(10.8-167.1)

MØ, 10^6/ml., collected 1-10 days post injury from 17 representative trauma patients and normal controls were cultured on matrix coated plastic with 20 ug/ml muramyl dipeptide (MDP) for twenty hours.

[a]TNFα activity levels in MØ supernates were measured in the LM cell bioassay.

[b]TNFα activity levels in MØ lysates were measured in the LM cell bioassay.

[c]Secreted and cell associated TNFα activity levels were combined for total TNFα level.

[d]Patients were separated into two groups based on total TNF_α protein production and cell associated vs secreted TNF_α production. Patients whose total MØ TNFα protein production was in the upper 50th percentil and whose cell associated TNF_α exceeded their secreted TNF_α were designated as Group 2. All others were designated as Group 1.

Table 2. Patients with Increased TNFα mRNA Levels and Comparable mRNA Persistence

Hours P.I.	Non Induced		MDP Induced	
	Norm	Pt	Norm	Pt
2	8.6	20.2	17.6	25.4
16-20	1.0	2.6	0.8	1.6
Residual	12%	13%	5%	6%
2	8.6	19.6	21.2	46.0
16-20	0.4	1.0	0.2	1.4
Residual	5%	5%	1%	3%
2	4.2	12.8	1.4	77.8
16-20	0.4	1.2	0.16	2.4
Residual	10%	9%	11%	3%
2	3.6	2.8	12.0	12.0
16-20	0.02	0.02	0.04	0.04
Residual	1%	1%	<1%	<1%
2	7.6	15.4	16.8	29.2
16-20	0.8	1.8	0.2	1.0
Residual	11%	12%	1%	3%
2	5.4	6.8	7.4	16.4
16-20	0.6	0.8	1.0	2.2
Residual	11%	12%	13%	13%
2	8.6	10.0	13.8	20.4
16-20	1.0	1.2	1.8	2.4
Residual	12%	12%	13%	12%
2	3.8	9.8	10.8	20.0
16-20	0.26	0.28	0.38	0.6
Residual	7%	3%	4%	3%
2	27.0	76.0	48.0	114.0
16-20	0.4	0.8	1.0	2.4
Residual	1%	1%	2%	2%
2	3.2	5.2	11.8	14.2
16-20	0.12	0.04	1.0	0.26
Residual	4%	1%	8%	2%
2	44.0	48.0	50.0	64.0
16-20	1.2	1.4	1.6	0.6
Residual	3%	3%	3%	1%

Results of 11 representative experiments from 115 conducted in which MØ from patients and normal controls were cultured for two or sixteen to twenty hours as in Fig. 2 with (MDP Induced) or without (Non Induced) addition of MDP. At the indicated time post induction (P.I.) cells were lysed and RNA content was analyzed as in Fig. 1. Sixteen or twenty hour TNF_α levels were compared to two hour levels and the residual percentage was calculated as 2 hr/16-20 hr levels.

Table 3. Patients With Increased TNFα mRNA Persistence

Hours P.I.	Non Induced		MDP Induced		Patient
	Norm	Pt	Norm	Pt	Outcome
2	10.0	17.8	14.2	45.6	Expired
16-20	1.6	5.8	0.6	7.8	ARDS
Residual	16%	33%	4%	17%	MOF
2	9.8	3.8	19.6	19.6	Expired
16-20	0.06	0.08	0.08	0.6	SIRS
Residual	0.6%	2%	0.4%	3.0%	
2	4.8	5.8	9.8	21.6	Recovered
16-20	0.2	0.4	0.2	3.2	Sepsis.
Residual	4.2%	6.8%	2.0%	14.8%	SIRS
2	6.2	20.8	10.0	31.6	Recovered
16-20	0.6	2.8	0.4	3.8	Pneumococcal
Residual	9.7%	13%	4%	12%	Pneumonia,
2	14.6	26.8	20.4	68.6	Recovered
16-20	0.8	17.6	1.0	33.4	Sepsis
Residual	6%	65%	5%	49%	Pneumonia
2	8.4	13.0	17.8	32.4	Recovered
16-20	0.4	3.6	0.4	5.8	Pneumococcal
Residual	5%	16%	2%	18%	Pneumonia

Results of 6 representative experiments from 10 conducted in which MØ from 7 patients (Pt) determined to have increased TNF_α mRNA persistence and normal controls (Norm) were cultured for two or sixteen to twenty hours either with (MDP Induced) or without (Non Induced) addition of MDP. Cells were lysed and RNA was analyzed as in Fig. 1. Residual percentage was calculated as 2 hr/16-20 hr TNF_α levels.

Table 4. Increased TNF$_\alpha$ mRNA Stability In Patients' MØ Treated With Actinomycin D

	mRNA in attomoles 10^6 monocytes (% residual)							
Hours [a]	**Unstim.** [b]		**U/Act.D** [c]		**MDP** [d]		**MDP/Act.D** [e]	
P.I.	**Nor.**	**Pt.**	**Nor.**	**Pt.**	**Nor.**	**Pt.**	**Nor.**	**Pt.**
Experiment 1								
2	5.2	10.2	5.2	10.2	10.2	18.2	10.2	18.2
6	3.8	11.4	0.8	9.4	7.6	20.8	0.4	13.2
	(79%)[f]	(112%)	(16%)	(92%)	(75%)	(114%)	(4%)	(73%)
16	1.0	4.0	0.4	3.4	0.8	5.6	0.007	6.1
	(19%)	(39%)	(7%)	(33%)	(8%)	(31%)	(1%)	(33%)
Experiment 2								
2	6.0	3.4	6.0	3.4	36.0	5.4	36.0	5.4
6	2.0	1.6	2.2	1.26	12.0	18.0	5.8	2.2
	(33%)	(47%)	(37%)	(37%)	(33%)	(333%)	(16%)	(41%)
Experiment 3								
2	3.9	4.1	3.9	4.1	6.0	7.0	6.0	7.0
16	0.3	0.4	0.7	0.5	0.4	0.6	0.1	4.2
	(8%)	(10%)	(18%)	(12%)	(7%)	(9%)	(2%)	(60%)
Experiment 4								
2	3.9	1.3	3.9	1.3	6.0	2.1	6.0	2.1
6	2.8	0.6	2.1	1.2	0.7	2.6	0.5	0.9
	(72%)	(46%)	(64%)	(92%)	(12%)	(124%)	(8%)	(44%)
16	0.3	0.2	0.7	0.4	0.4	0.3	0.1	0.4
	(8%)	(16%)	(18%)	(31%)	(7%)	(14%)	(2%)	(19%)

MØ from two patients (Pt.) and normal controls (Nor.) were cultured as indicated. RNA content was analyzed as in Fig. 1. [a] time MØ were cultured subsequent to MDP induction; [b] MØ were cultured as in Fig.2 without addition of MDP; [c] Actinomycin D (1-ug/ml)was added to culture at 2 hours P.I.; [d] MDP (20ug/ml) was added to cultures; [e] Actinomycin D was added as above to MDP induced cultures; [f] Residual percentage was calculated as 2 hr/6 hr or 16 hr TNF$_\alpha$ mRNA levels.

Table 5. Comparison of patients with high and low TNFα mRNA residual content.

Residual [a]	Increased Total TNFα Protein [b]	Increased Cell Associated TNFα Protein [c]	Clinical Comp [d]
Low (n=28)	**90%**	**32%**	**7%**
High (n=7)	**100%**	**100%**	**100%**

TNFα mRNA residual level = 16-20 hr / 2 hr TNF_{α} mRNA levels. [a] High residual patients >2 fold normal MDP induced residual and > non induced normal residual. [b] Patients were classified as having increased total TNFα protein if the patient level was at least twice that of concomitantly assayed normal levels. [c] Patients were classified as having increased cell associated TNFα protein if the cell associated TNFα level was greater than the secreted level. [d] Patients were classified as having clinical complications if MOF, systemic infection, or pseudomonas pneumonia occurred.

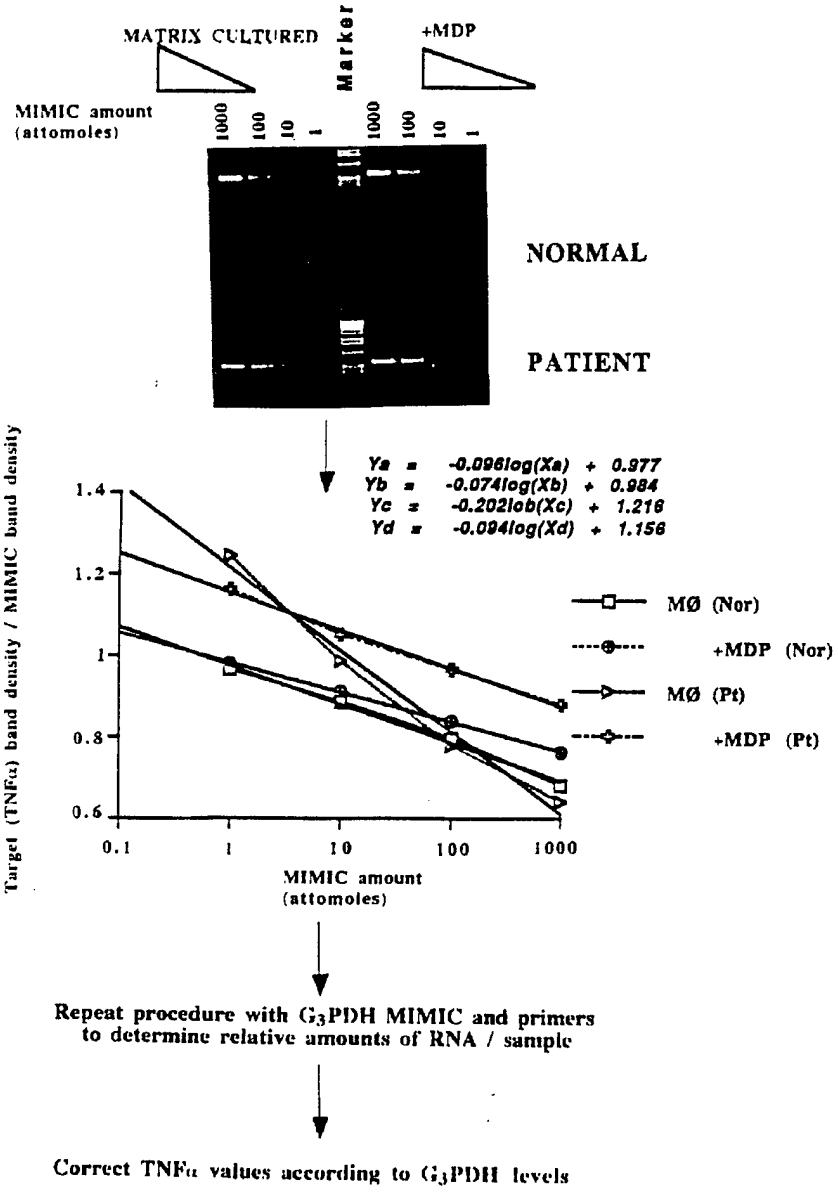
Figure 1. Procedure for quantitative measurement of mRNA species. Normals' or patients' monocytes were matrix cultured alone (matrix cultured) and in the presence of muramyl dipeptide (MDP). The first cycle of TNF_α specific PCR/electrophoresis/densitometry/ratio analysis was followed by a second cycle of G_3PDH specific PCR/electrophoresis/densitometry/ratio analysis. The G_3PDH results facilitated correction of the TNF_α levels. A broad range of Mimic amounts were used to clearly illustrate the procedure. In subsequent experiments, Mimic in the range of 2.5 - 0.02 attomoles were used.

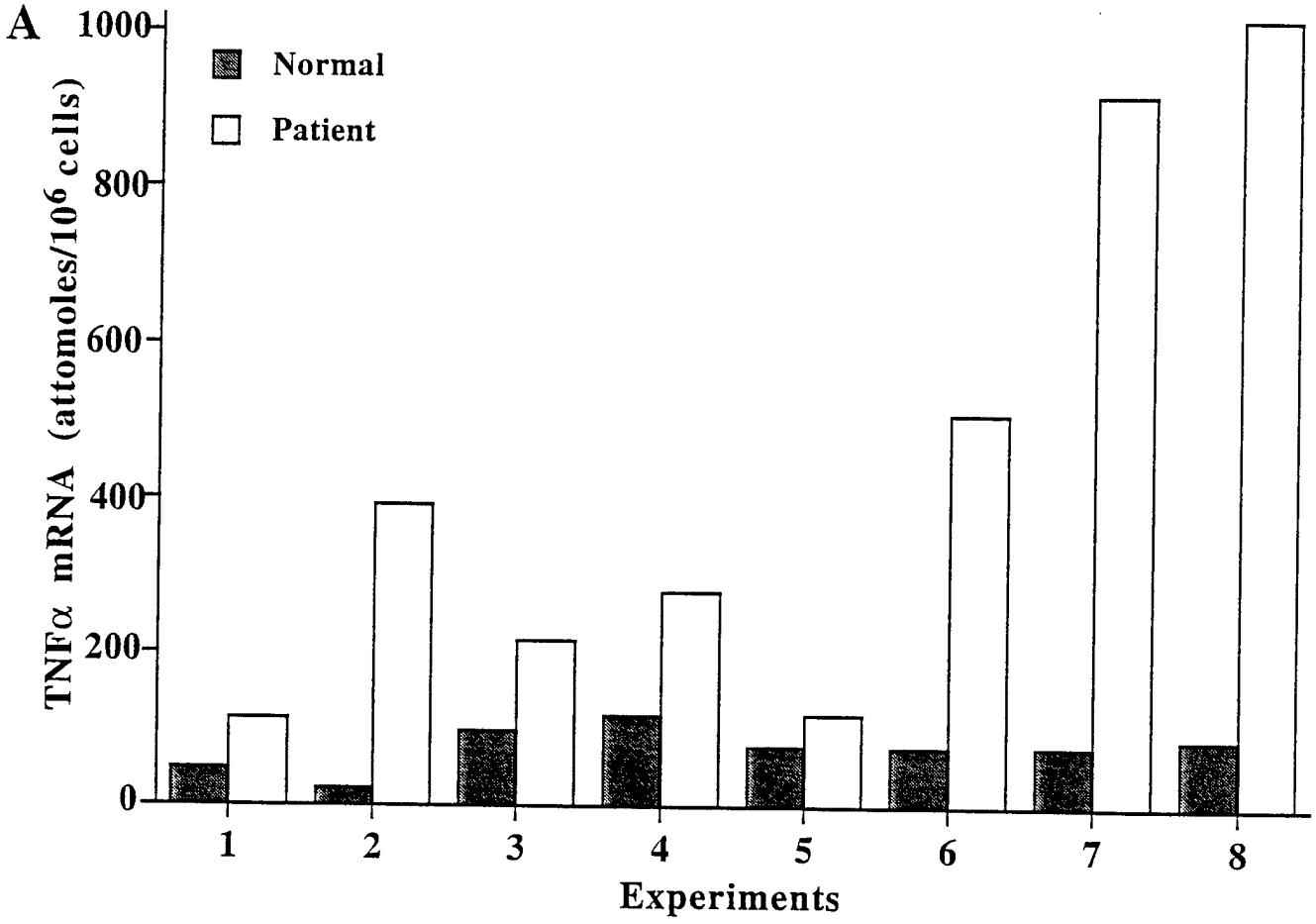
Figure 2. Increased TNF_α mRNA levels in patients' MØ. A). MØ from 8 representative patients and normal controls were cultured as in Fig. 2. Cells were lysed and whole cell RNA was reverse transcribed and amplified in competitive mimic PCR to determine TNF_α specific RNA levels. Reported TNF_α levels were normalized according to G_3PDH levels for each sample. Difference between normals' and patients' levels: p=0.0011. B). MØ were cultured and mRNA was analyzed as above with the exception that no MDP was added to the culture. Difference between normals' and patients' levels: p= 0.0742.

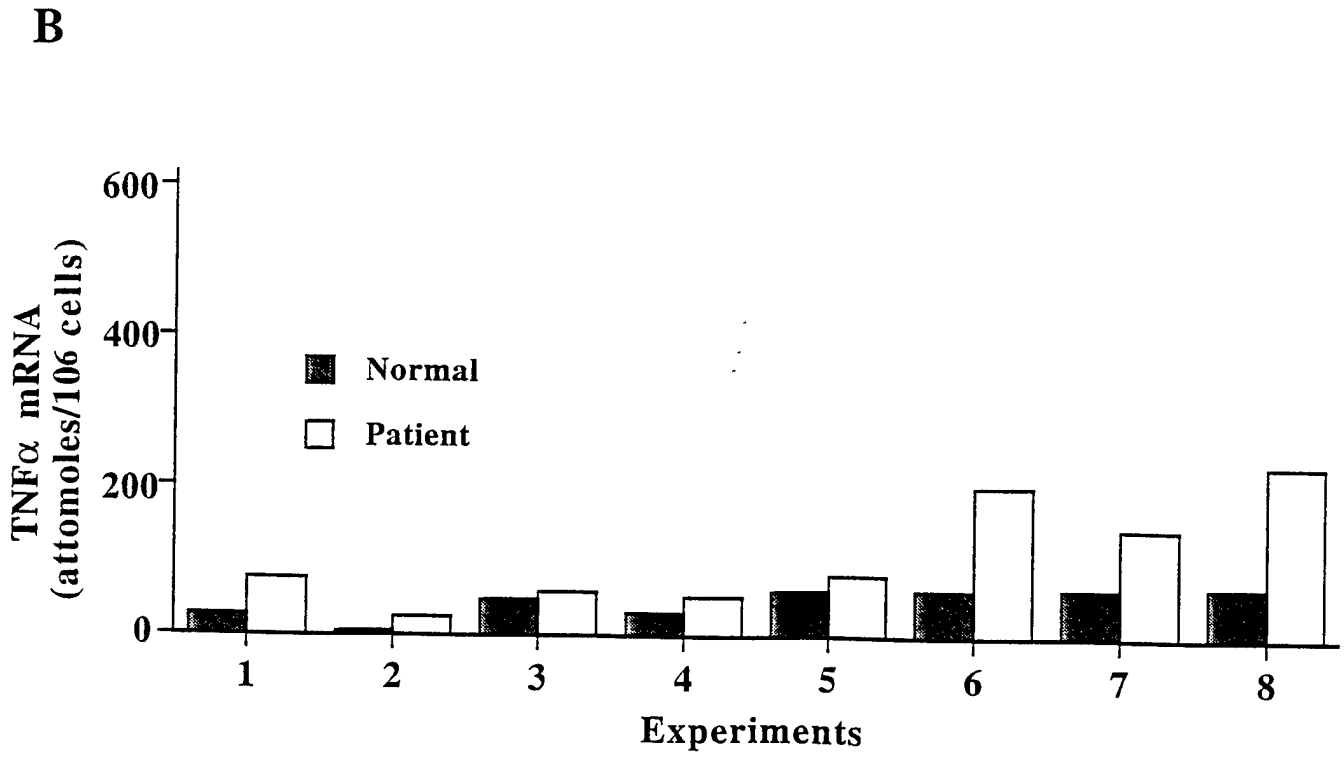
Figure 3. Varied degrees of augmentation of TNF_α mRNA in MDP induced patients' MØ. Monocytes from 10 representative patients and normal controls were cultured as in Fig. 2 with (MDP) or without (Cul) addition of MDP. Cells were lysed and RNA was analyzed as in Fig. 1. *Inset:* Results from normals' monocytes on an expanded scale to show increases in TNF_α levels subsequent to MDP treatment.

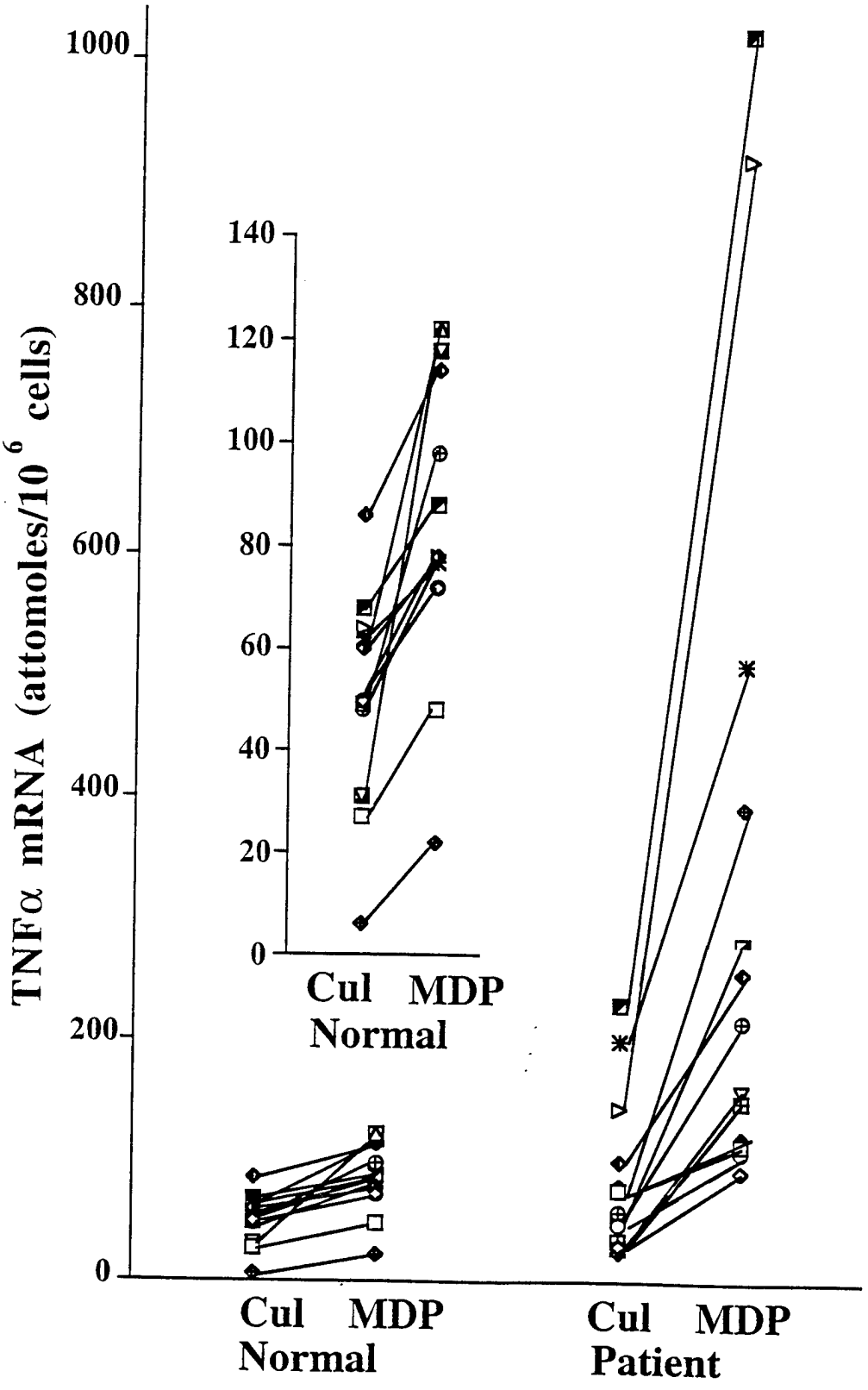
Figure 4. Increased TNF_α mRNA in patients' MØ after 20 hours. Monocytes from a hyperaccumulating patient and a normal control were cultured for two or twenty hours as in Fig. 2 with (MDP) or without addition of MDP. MØ were lysed and RNA was isolated and reverse transcribed. cDNA was analyzed as in Fig. 1. A). PCR was carried out using TNF_α specific primers and the TNF_α Mimic at indicated concentrations as described in Methods. B). PCR was carried out using G_3PDH specific primers and the G_3PDH Mimic as described in Methods. Results from G_3PDH assays were used to normalize results from TNF_α assays.

Figure 5. Patient MØ with increased TNF_α mRNA stability in Actinomycin D treated cultures. MØ from a hyperaccumulating patient and a normal control were cultured for two or six hours as in Fig. 2 with or without addition of MDP. At two hours post induction, 1μg/ml actinomycin D was added to indicated cultures. At indicated times post MDP induction, cells were lysed and RNA was analyzed as in Fig. 6. A). PCR was carried out using TNF_α specific primers and the TNF_α Mimic as described in Methods. B). PCR was carried out using G_3PDH specific primers and the G_3PDH Mimic as described in Methods.


MATRIX CULTURED
Marker
+MDP
MIMIC amount (attomoles)
1000
100
10
–
1000
100
10
–
NORMAL
PATIENT
Ya = -0.096log(Xa) + 0.977
Yb = -0.074log(Xb) + 0.984
Yc = -0.202lob(Xc) + 1.218
Yd = -0.094log(Xd) + 1.156
Target (TNFα) band density / MIMIC band density
1.4
1.2
1
0.8
0.6
0.1
1
10
100
1000
MIMIC amount (attomoles)
MØ (Nor)
+MDP (Nor)
MØ (Pt)
+MDP (Pt)
Repeat procedure with G3PDH MIMIC and primers to determine relative amounts of RNA / sample
Correct TNFα values according to G3PDH levels


A
1000
800
600
400
200
0
Normal
Patient
TNFα mRNA (attomoles/10^6 cells)
1
2
3
4
5
6
7
8
Experiments


B
600
400
200
0
Normal
Patient
TNFα mRNA
(attomoles/106 cells)
1
2
3
4
5
6
7
8
Experiments


1000
800
600
400
200
0
TNFα mRNA (attomoles/10^6 cells)
140
120
100
80
60
40
20
0
Cul MDP
Normal
Cul MDP
Normal
Cul MDP
Patient

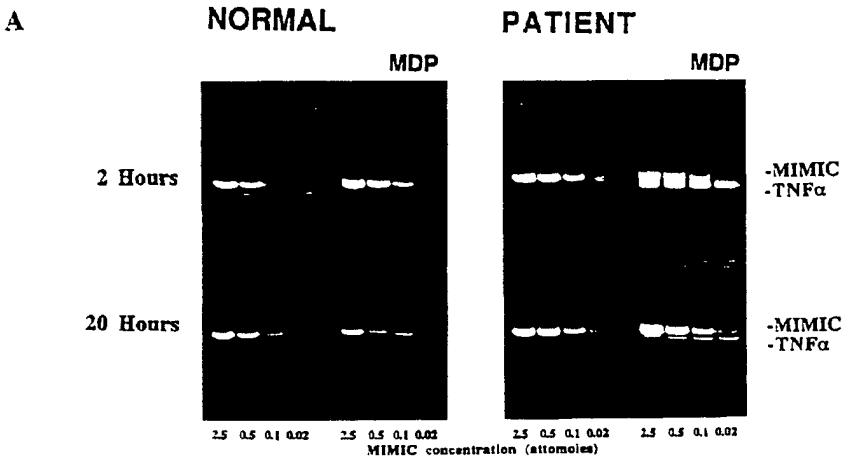
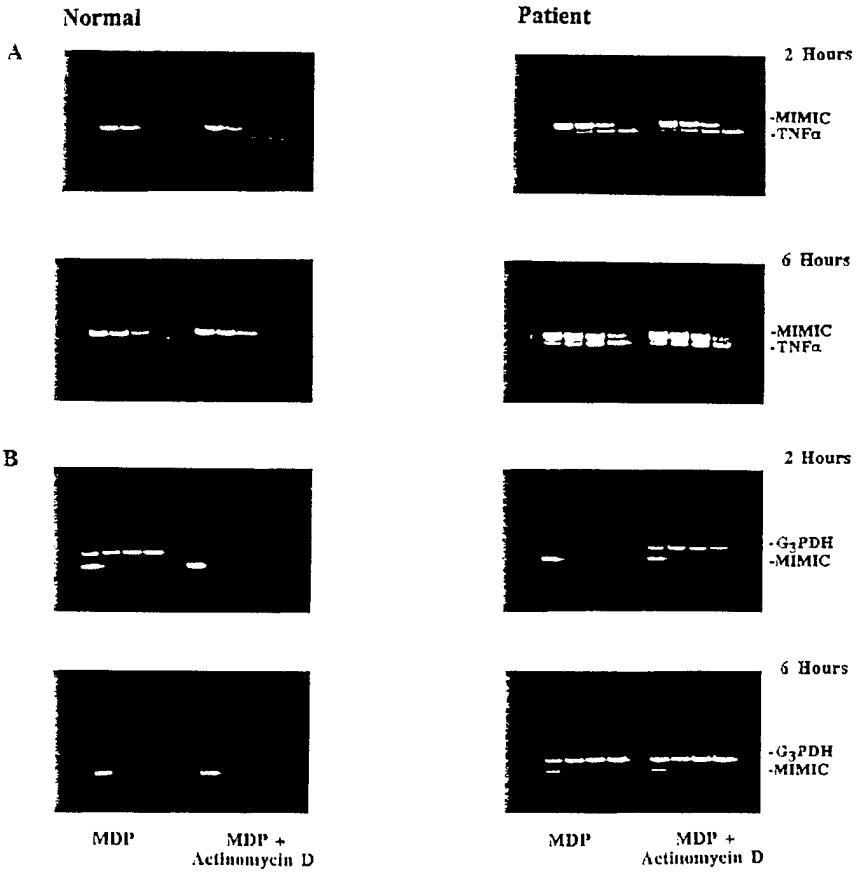

A
NORMAL
PATIENT
MDP
MDP
2 Hours
20 Hours
-MIMIC
-TNFα
-MIMIC
-TNFα
2.5 0.5 0.1 0.02 2.5 0.5 0.1 0.02
2.5 0.5 0.1 0.02 2.5 0.5 0.1 0.02
MIMIC concentration (attomoles)
B
2 Hours
20 Hours
-G3PDH
-MIMIC
-G3PDH
-MIMIC

Figure 5


Normal
Patient
A
2 Hours
-MIMIC
-TNFα
6 Hours
-MIMIC
-TNFα
B
2 Hours
-G3PDH
-MIMIC
6 Hours
-G3PDH
-MIMIC
MDP
MDP + Actinomycin D
MDP
MDP + Actinomycin D

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Altered IL-10 Levels in Trauma Patients' MØ and T Lymphocytes

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Trauma results in concomitant immunosuppression and elevated monocyte (MØ) inflammatory cytokine levels. The augmenting or ameliorating effect of IL-10 in septic complications after trauma is controversial. Here, IL-10 levels of trauma patients' and normals' PBMC, isolated MØ, and isolated T cells were assessed and correlated to their PBMC mitogen responses, their T-cell proliferation in an APC independent system, and their MØ production of elevated TNF-α levels. Trauma patients with depressed PBMC responses to PHA stimulation also had significantly decreased IL-10 levels in their stimulated PBMC supernates ($P = 0.0022$) and their MDP-stimulated isolated MØ population ($P = 0.0004$). However, patients with depressed PHA responses could have either normal or depressed T-cell proliferation in an anti-CD3-, anti-CD4-stimulated system. If APC-independent T-cell proliferation was depressed, induced IL-10 levels were suppressed ($P = 0.007$). However, if APC-independent T-cell proliferation was normal or elevated, IL-10 levels could be normal or elevated ($P = 0.018$). Decreased IL-10 levels correlated with depressed mitogen responses and depressed T-cell proliferation. IL-10, therefore, could not be inducing trauma patients' immunosuppression. Patients with elevated MØ TNF-α levels had depressed MØ IL-10 levels.

KEY WORDS: IL-10; trauma patients; monocytes; T cells.

INTRODUCTION

Mortality after severe trauma is often delayed and results from lung or kidney failure rather than from the injury itself (1). Increased, deregulated cytokine production, particularly increases of TNF-α and IL-1, are proposed as mediating some of this posttrauma mortality (1–8). The trauma patients' exaggerated cytokine production has been attributed to repeated bacterial stimulation during the septic episodes that result from the patients' severe immunosuppression (2–4, 9). Depressed T-cell proliferation to mitogen and depressed IL-2 production have been hallmarks of trauma patients' immunosuppressed state (9–13). Recently, experiments using an *in vivo* murine system seem to suggest that excessive cytokine production in response to septic challenge could be reversed and mortality decreased by administration of the recently described cytokine IL-10 (14–16). In contradiction to these findings, other experiments in murine systems of hemorrhage-induced immunosuppression have reported increased splenocyte IL-10 production after injury and suggested excessive IL-10 production as responsible for postinjury immunosuppression in this murine system (17, 18).

IL-10 was originally described as a murine cytokine product of Th2 type clones that inhibited murine Th1 lymphokine production by decreasing monocyte (MØ) activation of Th1 cells (19, 20). Recent data demonstrated that monocytes/macrophage, B cells, $CD4^+$ Th1 clones, $CD8^+$ T cells, and mast cells can all produce various amounts of IL-10, which can then regulate a variety of lymphocyte and myeloid cell functions and suppress inflammatory cytokine production of T cells, monocytes/macrophage, and PMN (19–24). Although MØ appear to be the primary IL-10 source in stimulated normal human PBMC cultures, activated T cells may also be an important IL-10 source in trauma patients (21, 25–27). The data demonstrating that human IL-10 was a potent suppressor of human T-cell proliferation, both directly in a MØ independent system, and indirectly through its action on APC, suggest that IL-10 may be elevated after injury and responsible for posttrauma immunosuppression (19, 27–29). In contrast, the ability of IL-10 to down-regulate MØ, T cells, and PMN proinflammatory cytokines supports the hypothesis that IL-10 levels are inadequate after injury and that their

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increase may be beneficial (14, 19, 22–25, 30). In the present study, we have examined PBMC, MØ, and T-cell IL-10 production by trauma patients who also are severely immunosuppressed and who may also have hyperelevated MØ TNF-α levels. IL-10 levels produced by these selected trauma patients' T lymphocytes and MØ are compared to IL-10 levels produced by trauma patients without severe immunosuppression and to paired normal controls.

MATERIALS AND METHODS

Patient Population. A total of 31 patients admitted to the University of Massachusetts Medical Center Trauma Unit, Worcester, was included in this study. Fifteen patients with mechanical trauma (injury severity score >30) and 16 patients with thermal trauma (>20% total-body surface burns) were assessed. Their ages ranged from 19 to 85; the median age was 35. There were 23 men and eight women. Normal controls were tested along with each patient. Volunteers from laboratory and hospital staff at the University of Massachusetts Medical Center (ages 20–58) served as normal controls. Informed consent was obtained from all patients and controls and the study was approved by the Institutional Review Board.

Cytokines. Recombinant human interleukin-10 (IL-10) was a generous gift from Schering-Plough Research Institute, Kenilworth, NJ. Recombinant human tumor necrosis factor alpha (TNF-α) was obtained from Collaborative Biomedical Products, Bedford, MA. Recombinant human transforming growth factor beta (TGF-β) was generously provided by Genentech, Inc., South San Francisco, CA, supplied in 20 m*M* NaAc, pH 5.0. Activity of TGF-β was confirmed in the sensitive mink lung (MvLu) bioassay.

Monocyte Separation and Stimulation. Monocytes from patients' and normals' blood were isolated from Ficoll-Hypaque gradient-separated mononuclear cells (PBMC) by selective adherence as described previously (31). Briefly, nonadherent cells were removed after 1.5 hr of adherence to microexudate-treated plastic surface, resulting in a >95% MØ purity as determined by fluorescein isothiocyanate (FITC) -labeled OKM5 staining. FcγRI cross-linked populations were obtained from normals by rosetting the MØ with human O, RhO(D)$^+$ erythrocytes (Selectogen, Ortho Diagnostic System, Inc., Raritan, NJ), and treated with anti-RhO(D) human immunoglobulin (RhO-GAM, Ortho Diagnostic System) as previously described (32, 33). This rosetting technique provides cross-linking stimulation of the high-density FcγRI-bearing (FcγRI$^+$) MØ subpopulations and yields an enriched FcγRI$^+$ population (33, 34). Three million MØ per well were cultured in 3.0 ml of RPMI 1640 medium (JRH Biosciences, Lenexa, KS), supplemented with 15% FBS (Sigma Chemical Co., St. Louis, MO), 50 units/ml penicillin-G, 50 μg/ml streptomycin, 50 μg/ml gentamicin, 2.5 μg/ml fungizone, 4 m*M* L-glutamine, 1 m*M* Na pyruvate, and 1% minimal essential medium (MEM) nonessential amino acids (JRH Biosciences). Endotoxin contamination was less than 15 pg/ml in the culture media and FBS, and all media contained 100 units/ml polymyxin B sulfate (GIBCO Laboratories, Grand Island, NY).

Normals' and patients' MØ were stimulated with 20 μg/ml MDP, a gram-positive cell wall analog (compound CGP 11637 was generously provided by CIBA-Geigy, Basel, Switzerland). Recombinant human IL-10 (50 units/ml) was added, along with MDP, in a number of experiments with normals' MØ. In some of the experiments with normals' MØ, 200 units/ml recombinant human TNF-α was added either alone or in combination with 20 μg/ml MDP. Similarly, 2.4 ng or 4.8 ng/ml TGF-β was added along with 20 μg/ml MDP in some experiments. MØ supernates were collected after 16–18 hr of stimulation and kept frozen at −80°C until the cytokine assays were performed. Adherent MØ were collected by EDTA treatment and scraping. Recovered cells were kept frozen at 5×10^6/ml concentration in PBS for further analysis.

Mitogen Assays. PBMC (2×10^5 cells/200 μl/well) were cultured in flat-bottomed microtiter plates (Becton Dickinson, Lincoln Park, NJ) in presence or absence of PHA (Murex Diagnostics Ltd., Dartford, England). The experiments were set up in duplicate. In one set, supernates were harvested after 30 hr and stored at −80°C until the day of assay for IL-10. In another set, cells were cultured for 72 hr for proliferation assays by [^{3}H]thymidine incorporation at the last 18 hr of incubation.

T-Cell Purification and Stimulation. PBMC T cells were purified from PBMC as previously described (32, 35). Briefly, normals' and patients' PBMC were depleted of MØ by selective adherence to microexudate-treated plastic surfaces. Nonadherent cells were rosetted with neuraminidase (Sigma) -treated sheep red blood cells (SRBC). The SRBC-rosetted cells were >90% T cells with <1% contamination by B cells or monocytes, as determined by flow cytometric analysis. The purified T cells were cultured (2×10^5 cells/200 μl/well) in flat-bottomed microtiter plates in presence of immobilized anti-CD3 and anti-CD4. MAb were immobilized onto plastic microtiter plates as described (36). In brief, anti-CD3 (Boehringer Mannheim, Indianapolis, IN), diluted in RPMI 1640 was placed (1.5 μg/50 μl/well) in

each of the wells of 96-well flat-bottomed microtiter plates (Becton Dickinson), incubated at room temperature for 1.5 hr, and then washed with PBS two times to remove nonadherent MAb. The process was repeated with anti-CD4 (Biosource International, Camarillo, CA) (1 μg/50 μl/well). After 24 hr of culture, 100 μl of supernates were harvested from each well and replenished with 100 μl of fresh medium and continued for proliferation for another 48 hr in a [^{3}H]thymidine incorporation assay.

TNFα Bioassay. TNF-α activity in MØ supernates (secreted TNF-α) and sonicated MØ lysates (cell-associated TNF-α) were measured in the L–M cell bioassay as previously described (33). Both cell-associated and secreted MØ TNF-α activity were totally inhibited by anti-TNF-α neutralizing antibody.

IL-10 ELISA. IL-10 levels in the supernates of PBMC, MØ, and T cells were determined by a specific ELISA kit (Biosource International) according to the protocol recommended by the manufacturer. The sensitivity of the assay was 5 pg/ml.

Statistical Analysis. Because of the known individual genetic variation in cytokine levels, parametric statistical analysis (i.e., mean and standard deviation) is inappropriate. Data for normals and patients in each assay are paired, but the individual samples analyzed in a Wilcoxon nonparametric assay (Macintosh Statview) treat each individual as coming from an independent population.

RESULTS

Examination of Trauma Patients' PBMC and MØ Supernates for IL-10 Levels. The depressed mitogen responses that typify burn and trauma patients have been suggested as resulting from increased PGE_2 levels, decreased IL-2 production, increased TGF-β levels, and/or depletion of T-lymphocyte numbers (7–12, 18, 37). IL-10 levels have also been suggested as being increased after severe hemorrhage and possibly mediating post-trauma immunosuppression (17, 18). Since IL-10 has been shown to depress costimulation of T-cell proliferation and to directly decrease T-lymphocyte IL-2 levels, it seemed reasonable that IL-10 levels might be increased in the PBMC supernates of trauma patients whose PHA mitogen-induced proliferation is depressed (19, 27–29). However, when the supernates from PHA-stimulated immunosuppressed patients' PBMC were assayed for IL-10 levels by ELISA and compared to simultaneously stimulated assayed normals' PBMC supernates, the levels of IL-10 were significantly ($P = 0.002$ Wilcoxon) depressed in the PHA-induced PBMC supernates of patients who were concomitantly experiencing decreased mitogen responses (Table I). The surprising finding that IL-10 levels were depressed in these immunosuppressed patients' PBMC led us to examine isolated MØ production of IL-10 in trauma patients who experienced depressed PHA responses. MØ are the primary producers of IL-10 in the freshly isolated and stimulated human PBMC population (25, 26). It was possible that excessive production of T lymphokines in the PBMC populations was decreasing the MØ IL-10 production or that an early *in vivo* stimulated increase of IL-10 in the patients' PBMC was autodepressing their subsequent *in vitro* MØ IL-10 production (22).

We examined the IL-10 production of isolated patients' and normals' MØ either cultured alone for 16 hr or stimulated 16 hr with the bacterial cell wall analog MDP (20 μg/ml). When patients' MØ IL-10 levels were assessed sequentially over time after injury, the IL-10 levels were seen initially to be in the normal range, then decrease dramatically at five to nine days after injury (Fig. 1). IL-10 levels would then begin to renormalize at later postinjury periods in recovering patients (Fig. 1). Consequently, individual patients' MØ IL-10 levels were repeatedly assessed at three to four day intervals over their clinical course and treated as independent samples. A particular patient's MØ IL-10 data and corresponding mitogen response might be normal, depressed, or elevated, depending on the postinjury day on which the sample was collected. As can be seen in Table II, those trauma patients whose PBMC responses were suppressed also exhibited significant reduction in their IL-10 levels either from MØ cultured alone or from MDP-stimulated MØ when compared to controls. In fact, when 37 samples of MØ supernates collected from 24 mitogen-depressed patients at different postinjury days were assayed for IL-10 levels, all patient samples showed a statistically significant ($P = 0.0001$ Wilcoxon) depression in both unstimulated and stimulated levels of MØ IL-10 as compared to their paired controls.

As illustrated by the six representative experiments in Fig. 2, there was significant variation in the assayed levels of IL-10 for the normals' MØ. However, in all 37 assays, the level of IL-10 produced by the patient MØ sample was depressed as compared to paired normal's. The median levels of IL-10 in cultured and MDP-stimulated normals' MØ samples were 0.910 and 2.553 ng/10^6 MØ/ml, respectively. The corresponding median IL-10 levels for the unstimulated and stimulated trauma patients' MØ were 0.128 and 0.461 ng/10^6 MØ/ml, showing significant suppression ($P = 0.0001$) when the entire group was analyzed with the Wilcoxon nonparametric test. We also considered the possibility

Table I. Depressed IL-10 Production by Immunosuppressed Trauma Patients' PBMC

Experiment	% decrease proliferation[a]	IL-10 (ng/10^6 PBMC/ml)[b] Normal	Patient[c]
1	41	5.350	1.160
2	65	4.005	1.850
3	81	2.850	0.320
4	42	3.600	<0.005
5	96	2.040	0.460
6	66	4.520	0.380
7	57	4.080	0.930
8	98	4.600	<0.005
9	85	5.100	0.360
10	71	2.900	1.470
11	71	4.600	0.790
12	67	1.640	0.340
		$P = 0.0022$[d]	

[a] PHA-induced proliferation calculated from [^{3}H]thymidine incorporation using the formula $A - B/A \times 100$ where A = counts for normal, B = counts for patient; and the patients having >30% decrease in proliferation were considered immunosuppressed.

[b] PBMC were cultured in 96-well plates (2×10^5 cells/200 μl/well) in the presence of PHA. After 24 hr of culture, supernates were harvested and assessed for IL-10 by ELISA.

[c] Twelve samples were collected from six patients at different postinjury days.

[d] Statistical significance (P) between normal and patient values determined by Wilcoxon nonparametric test.

that early elevated MØ IL-10 levels stimulated *in vivo* by the trauma might be suppressing subsequent IL-10 production *in vitro*, since IL-10 is autosuppressive (22). We examined three patients' and paired normals' MØ IL-10 levels at both 3 h and 16 hr after culture. The 3-hr MØ IL-10 levels of the patients were unmeasurable, as were the normals' IL-10 levels. After 16 hr, the normals' MØ produced measurable IL-10 levels, while the patients' MØ IL-10 levels were significantly decreased (data not shown). These data suggest that early *in vitro* MØ production of IL-10 is not responsible for decreasing IL-10 levels at the time we assay. In addition, the data show that MØ-produced IL-10 is not responsible for the decreased mitogen-induced proliferation of these trauma patients since IL-10 levels are depressed in these patients.

Examination of T-Cell IL-10 in Immunosuppressed Patients and Normals. MØ are the primary IL-10 source in stimulated human PBMC (19, 25, 26). Consequently, the decreased MØ IL-10 levels in the PHA-stimulated supernates from patients' PBMC might mask decreased or increased IL-10 production by the patients' T cells. Since T-cell numbers are depleted after injury, the assay of PBMC supernates might fail to detect altered IL-10 levels in the remaining T-cell population. In view of reports of increased IL-10 production in murine $CD4^+$ splenocytes posthemorrhage when the whole splenocyte population showed no change, the patients' isolated T lymphocytes also needed to be examined for IL-10 levels (17). We have previously shown that patients with depressed PBMC mitogen responses could display either

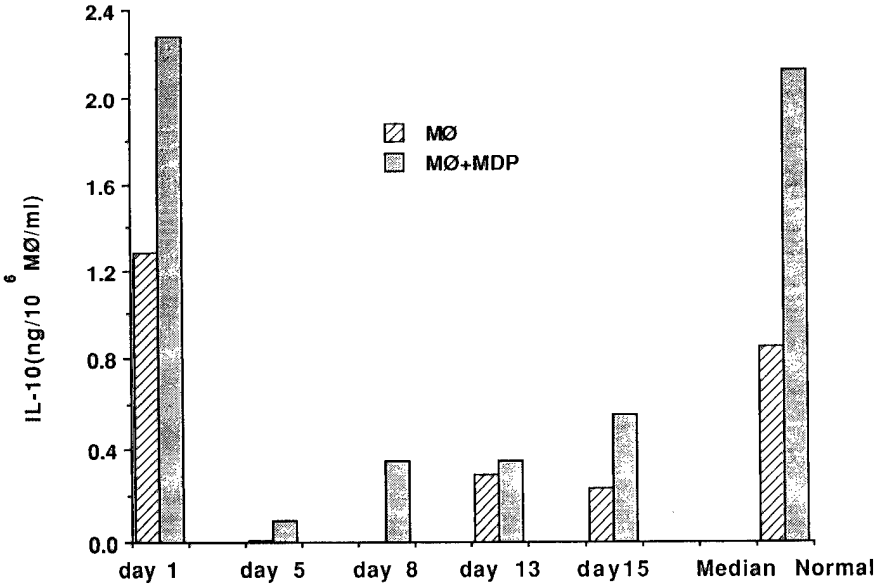

Fig. 1. MØ IL-10 levels of one patient at five different postinjury days. MØ from the patient and control normal were cultured (3×10^6 cells/3 ml) for 16 h in medium alone (MØ) or in the presence of MDP (20 μg/ml), and IL-10 levels in the culture supernates were measured by ELISA. Median values of MØ IL-10 levels of five control normals are included in the figure.

Table II. Depressed MØ IL-10 Production in Immunosuppressed Trauma Patients

Experiment	% decrease proliferation[a]	MØ IL-10 (ng/10^6-MØ/ml)[b] Unstimulated[c] Normal	Unstimulated[c] Patient[d]	MDP[e] Normal	MDP[e] Patient
1	39	2.775	0.031	4.687	0.075
2	91	3.186	<0.005	4.329	<0.005
3	73	0.621	0.083	2.451	0.134
4	67	0.265	<0.005	1.139	0.033
5	70	0.325	<0.005	1.022	<0.005
6	70	0.942	0.148	2.809	0.230
7	42	2.081	<0.005	3.024	0.099
8	51	0.480	0.013	1.313	0.096
9	72	0.623	<0.005	2.053	0.357
10	65	0.508	0.089	1.934	0.604
11	80	3.781	<0.005	10.466	0.461
12	55	3.559	0.686	6.195	0.700
13	79	0.860	<0.005	2.216	0.493
14	42	3.107	0.149	6.964	0.704
15	98	2.515	<0.005	5.944	<0.005
16	71	2.515	0.268	5.944	0.755
		$P = 0.0004$[f]		$P = 0.0004$[f]	

[a] PHA-induced proliferation calculated from [^{3}H]thymidine incorporation using the formula $A - B/A \times 100$ where A = counts for normal, B = counts for patient; and the patients having >30% decrease in proliferation were considered immunosuppressed.
[b] IL-10 levels in the MØ culture supernates measured by ELISA.
[c] MØ (3×10^6 cells/3 ml) cultured in medium alone for 16 hr.
[d] Sixteen samples collected from 12 patients at different postinjury days.
[e] MØ (3×10^6 cells/3 ml) cultured in medium and MDP (20 μg/ml) for 16 hr.
[f] Statistical significance (P) between normal and patient values determined by Wilcoxon nonparametric test.

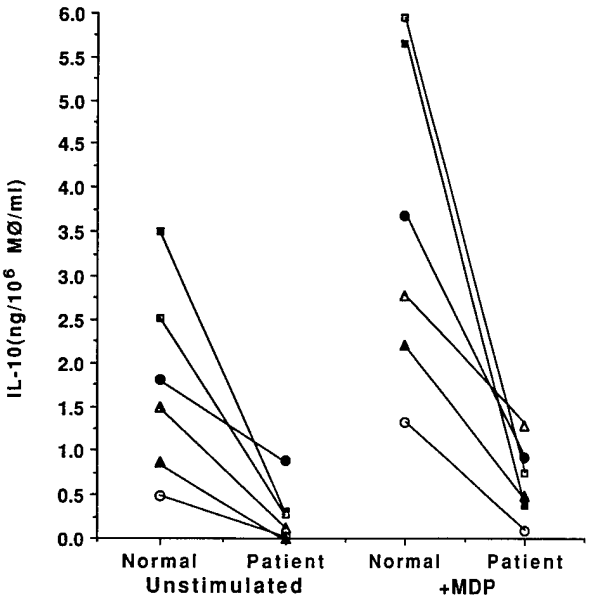

Fig. 2. Data of six representative experiments from 37 experiments, showing depressed MØ IL-10 production in patients (P value for normal unstimulated vs patient unstimulated = 0.0001 and for normal MDP vs patient MDP = 0.0001 for all the experiments, as assessed by Wilcoxon nonparametric test). MØ from both normals and trauma patients were cultured (3×10^6 cells/3 ml) for 16 hr in medium alone (unstimulated) or in the presence of MDP (20 μg/ml) and the IL-10 levels in the culture supernates were measured by ELISA.

depressed or normal T-cell proliferation in a MØ-independent T-cell proliferation system (38). We have also demonstrated that many immunocompetent trauma patients have highly elevated mitogen proliferation, and these patients also were found to have elevated T-cell proliferation (13). The trauma patients were, therefore, divided into three groups based on their altered mitogen and T cell proliferative responses. Patients with unaltered mitogen responses were not included. The groups are: patients with depressed mitogen responses and depressed T-cell proliferation, patients with depressed mitogen responses and normal or slightly elevated T-cell proliferation, and patients with elevated PHA and elevated T-cell proliferation. Patient and paired normal purified T cells from these three groups were assessed for IL-10 levels after stimulation by immobilized anti-CD3 and anti-CD4 (36, 38).

IL-10 has been previously shown to inhibit anti-CD3-stimulated human T-cell proliferation (28). Consequently, any suppressive effect of increased IL-10 produced by patients' T cells should be detectable in this MØ-independent T-cell proliferation assay. IL-10 levels were much lower in this MØ-independent T-cell proliferation system than in either PHA-stimulated PBMC or in MDP-stimulated isolated MØ. Nevertheless, as can be seen in Table III, patients with depressed mitogen responses and depressed T-cell proliferation in this T cell assay also had significantly depressed IL-10 production (Wilcoxon $P = 0.007$) as compared to the paired normals. These data indicate that increased T-cell production of IL-10 was not mediating decreases in the T-cell proliferation evidenced by these patients. All but one of the patients with depressed T-cell responses and depressed IL-10 levels went on to die. In striking contradiction, when we examined the T-cell levels of IL-10 produced by trauma patients with depressed mitogen responses, but normal or elevated T-cell proliferation, we found significantly elevated IL-10 production (Wilcoxon $P = 0.018$) as compared to the normals (Table IV). Elevated mitogen responses in trauma patients' PBMC have been previously attributed to a normal immune response to an ongoing bacterial and/or other *in vivo* stimulation (13). Patients with elevated mitogen responses had massively elevated T-cell proliferation and very elevated IL-10 levels (Table V). This IL-10 elevation in some immunocompetent trauma pa-

Table III. Depressed T Cell IL-10 Production in Trauma Patients with Depressed T Cell Proliferation[a]

Experiment	% decrease T cell proliferation	IL-10 (pg/10^6 T cells/ml)[b] Normal	Patient[c]
1	99	39	<5
2	97	117	<5
3	83	117	37
4	94	64	<5
5	60	108	<5
6	31	100	37
7	32	53	<5
8	56	156	<5
9	54	63	<5
		$P = 0.007$[d]	

[a] T cell proliferation was assessed in a [^{3}H]thymidine incorporation assay by culture of monocyte-depleted SRBC-rosetted T cells (2×10^5 cells/200 μl/well) in presence of immobilized anti-CD3 (1.5 μg/well) plus anti-CD4 (1 μg/well) for 72 hr. When the proliferation of T cells from the patient is decreased >30% as compared to the paired normal, it is considered depressed.

[b] IL-10 levels in the supernates of T cells cultured in presence of immobilized anti-CD3 (1.5 μg/well) plus anti-CD4 (1 μg/well) for 24 hr, were measured by ELISA.

[c] Nine samples were collected from four patients at different postinjury days.

[d] Statistical significance (*P*) between normal and patient values was determined by Wilcoxon nonparametric test.

Table IV. T Cell IL-10 Production in Trauma Patients with Normal or Elevated T Cell Proliferation[a]

Experiment	T cell proliferation (dpm × 10^{-3}) Normal	Patient	IL-10 (pg/10^6 T cells/ml)[b] Normal	Patient[c]
1	17	13	117	162
2	14	24	34	135
3	9	20	10	20
4	19	12	5	14
5	53	70	62	744
6	45	47	156	468
7	19	19	63	93
8	22	26	46	46
			$P = 0.018$[d]	

[a] T cell proliferation was assessed in a [^{3}H]thymidine incorporation assay by culture of monocyte-depleted SRBC-rosetted T cells (2×10^5 cells/200 μl/well) in presence of immobilized anti-CD3 (1.5 μg/well) plus anti-CD4 (1 μg/well) for 72 hr and expressed as dpm.

[b] IL-10 levels in the supernates of T cells cultured in presence of immobilized anti-CD3 (1.5 μg/well) plus anti-CD4 (1 μg/well) for 24 hr, were measured by ELISA.

[c] Eight samples were collected from five patients at different post injury days.

[d] Statistical significance (*P*) between normal and patient values was determined by Wilcoxon nonparametric test.

tients might reflect normally increased T-cell activity after *in vivo* stimulation. Even though the patients' PBMC mitogen response may be depressed, if their isolated T-cell proliferation is normal or slightly elevated, then their IL-10 levels can be elevated. These data indicate that trauma patients' T cells have depressed, not elevated, IL-10 production when their T-cell proliferation is compromised, but can have elevated IL-10 production if their T cell proliferation is normal or elevated. In contrast, patients' MØ IL-10 production is always depressed when their PBMC mitogen responses are depressed.

Relationship of Elevated Patients' MØ TNF-α to IL-10 Levels. If MØ IL-10 levels are depressed after trauma in the face of the massive postinjury MØ stimulation by trauma-generated fibrin degradation products, complement split products, and bacterial stimuli, it could have profound effects on posttrauma production of monokines. MØ-produced IL-10 is suggested as playing a major down-regulatory role in normals' MØ TNF-α production and as contributing to the sharp peak and decline of TNF-α production after MØ stimulation (19, 22). TNF-α, IL-6, and PGE_2 are all known to be excessively elevated after trauma and to persist over a prolonged period after injury (2–7). We initially thought that the elevated TNF-α levels occurring after trauma might be suppressive to MØ production of IL-10. However, when we examined the effect of exogenous TNF-α addition to normal MØ, we found that IL-10 levels were actually increased by addition of TNF-α to the culture media (Fig. 3). Similar data were recently published showing TNF-α augmentation of normals' MØ IL-10 production (39). Since immunosuppressed trauma patients' MØ produced elevated TNF-α levels in response to MDP stimulation, we questioned what the IL-10 levels would be in MØ supernates from such patients. As illustrated in Table VI, those patients' MØ with hyperincreased TNF-α responses to MDP had simultaneously depressed IL-10 responses as compared to paired normals'. Although the level of TNF-α still detectable at 16 hr of culture with MDP is variable with different normals' MØ, their IL-10 levels are always stimulated. In contrast, those patients' MØ that show persistently high TNF-α levels after 16 hr of MDP stimulation show significantly depressed IL-10 levels. These data suggest that patients' MØ with exaggerated TNF-α responses may also fail to produce IL-10 and consequently fail to rapidly down-regulate their TNF-α levels after activation.

Sensitivity of Patients' MØ TNF-α to IL-10 Down-Regulation

A recent report suggested that plasma IL-10 levels were elevated, not depressed, in patients with septic shock (40). Although these septic patients were not

Table V. Increased Mitogen Induction and T-Cell Proliferation Concomitant to Increased IL-10

Experiment	% increase PHA response[a]	T cell proliferation (dpm × 10^{-3})[b] Normal	Patient[c]	IL-10 (pg/10^6 T cells/ml)[d] Normal	Patient
1	485	49	104	122	175
2	100	17	47	8	94
3	89	9	22	10	59
4	187	19	25	88	660
5	65	11	23	20	41
6	40	10	56	26	76
7	80	22	150	9	175
8	89	18	65	8	48
		$P = 0.0117$[e]		$P = 0.0117$[e]	

[a] PHA-induced proliferation calculated from [^{3}H]thymidine incorporation using the formula $B - A/A \times 100$ where A = baseline proliferation of patient's PBMC measured by day 1 after injury and B = proliferation of PBMC of the same patient corresponding to the day of assessment of T-cell proliferation and IL-10 production.

[b] T-cell proliferation was assessed in a [^{3}H]thymidine incorporation assay by culture of monocyte-depleted SRBC-rosetted T cells (2×10^5 cells/200 μl/well) in presence of immobilized anti-CD3 (1.5 μg/well) plus anti-CD4 (1 μg/well) for 72 hr and expressed as dpm.

[c] Eight samples were collected from three patients at different postinjury days.

[d] IL-10 levels in the supernates of T cells cultured in presence of immobilized anti-CD3 (1.5 μg/well) plus anti-CD4 (1 μg/well) for 24 hr, were measured by ELISA.

[e] Statistical significance (P) between normal and patient values was determined by Wilcoxon nonparametric test.

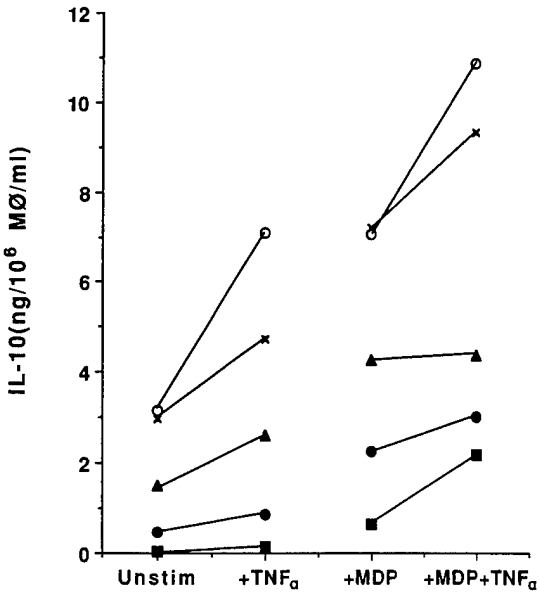

Fig. 3. Data of five representative experiments from 10 experiments, showing induction of MØ IL-10 production of TNF-α (P value for unstimulated vs TNF-α-stimulated = 0.018 and for MDP vs MDP + TNF-α = 0.0117 for all the experiments, as assessed by Wilcoxon nonparametric test). MØ from normals were cultured (3×10^6 cells/3 ml) for 16 hr in medium alone (unstimulated), MDP (20 μg/ml), TNF-α (200 units/ml), or MDP (20 μg/ml) in presence of TNF-α (200 units/ml) and the IL-10 levels in the culture supernates were measured by ELISA.

immunosuppressed trauma patients, these data suggested that elevated MØ TNF-α production might be persisting in the presence of high IL-10 production after trauma. MØ TNF-α production after trauma, has been described as resistant to down-regulation by PGE_2 (7, 8). In addition, IL-10 down-regulation of monokines has also been reported to vary depending on the inducing stimuli (41). Consequently, trauma patients' MØ production of TNF-α in response to nonbacterial stimuli might be insensitive to IL-10 down-regulation, thereby providing an additional explanation for elevated MØ TNF-α production after injury. Exogenous recombinant human IL-10 was added to cultures of patients' and normals' MØ at 50 units/ml. We have previously shown that this IL-10 concentration suppresses >95% of the normal MØ levels of TNF-α produced in response to MDP stimulation (data not shown). In this set of experiments, paired normals' and immunosuppressed trauma patients' MØ were assessed for MDP-induced TNF-α production in the presence and absence of IL-10. As illustrated in Table VII, the elevated levels of TNF-α produced by the patients' MØ were still sensitive to the down-regulatory effects of IL-10, although in several cases significant TNF-α was still produced by patients' MØ in the presence of IL-10 addition. However, these patients' MØ had elevated TNF-α levels before MDP stimulation (data not shown). Since IL-10 can suppress TNF-α mRNA at both the transcriptional and translational levels, these data suggest that trauma patients' MØ *de novo* production of TNF-α in response to continued stimuli is still sensitive to IL-10 down-regulation (22, 42).

Effect of TGF-β on MØ IL-10 Production. In addition to TNF-α, trauma patients' MØ produce highly elevated levels of TGF-β (37). TGF-β is a potent down-regulator of a number of cytokines (35, 42). In a small series of experiments, we examined the effects of exogenous TGF-β on MØ IL-10 levels. As illustrated by five representative experiments of 13 (Fig. 4), addition of 2.4 ng/ml recombinant human TGF-β_1 to normal MØ cultures significantly depressed their IL-10 production (Wilcoxon $P = 0.0003$). Although these data indicate that TGF-β can depress normal MØ IL-10 levels, this mechanism may not be operative in the trauma patients where a variety of MØ stimuli are present in addition to bacterial challenge. We have previously presented data indicating that trauma patients' monokine production is stimulated by *in vivo* cross-linking of MØ FcγRI receptors by the massively increased IgG levels in these patients' circulation (43, 44). In addition, we and others

Table VI. Trauma Patients with Elevated MØ TNF-α Levels also Have Depressed MØ IL-10 Levels

Experiment	MØ TNF-α (ng/10^6 MØ/ml)[a]		MDP-induced MØ IL-10 (ng/10^6 MØ/ml)[c]	
	Normal	Patient[b]	Normal	Patient
1	0.847	5.755	2.094	0.728
2	1.154	11.603	5.660	0.393
3	<0.05	7.262	1.139	<0.005
4	8.342	68.875	2.842	0.348
5	8.103	28.586	2.555	<0.005
6	<0.05	4.813	10.466	0.461
7	4.915	10.167	2.216	0.493
8	6.125	12.372	6.964	0.704
9	<0.05	24.224	5.944	0.755
	$P = 0.0077$[d]		$P = 0.0077$[d]	

[a] TNF-α levels, cell-associated (MØ lysate) plus secreted (MØ supernates), of MØ cultured in medium and MDP (20 μg/ml) for 16 hr, were assessed by LM bioassays.
[b] Nine samples were collected from seven patients at different post injury days.
[c] IL-10 levels in the supernates of MØ cultured in medium and MDP (20 μg/ml) for 16 hr, were measured by ELISA.
[d] Statistical significance (*P*) between normal and patient values determined by Wilcoxon nonparametric test.

Table VII. IL-10 Down-Regulates MØ TNF-α Production in Normals and Patients

Experiment	MØ TNF-α (ng/10^6 MØ/ml)[a]			
	Normal		Patient[d]	
	MDP[b]	MDP + IL-10[c]	MDP	MDP + IL-10
1	5.65	<0.05	17.64	12.17
2	5.65	<0.05	20.2	5.4
3	2.37	<0.05	7.53	4.34
4	1.34	<0.05	35.6	4.23
5	12.36	6.25	22.57	16.90
6	1.26	<0.05	26.0	<0.05
7	3.97	0.78	8.33	1.05
8	4.82	1.43	11.13	2.86
9	2.97	<0.05	5.54	<0.05
	$P = 0.0076$[e]		$P = 0.0077$[e]	

[a] TNF-α levels, cell associated (MØ lysate) plus secreted (MØ supernates), were assessed by LM bioassays.
[b] MØ (3×10^6 cells/ml) were cultured in medium and MDP (20 μg/ml) for 16 hr.
[c] MØ (3×10^6 cells/3 ml) were cultured in medium, MDP (20 μg/ml) and IL-10 (50 units/ml) for 16 hr.
[d] Nine samples were collected from four patients at different postinjury days.
[e] Statistical significance (*P*) between MDP vs MDP + IL-10-stimulated TNF-α values was determined by Wilcoxon nonparametric test.

have shown that FcγRI cross-linking induction of normals' MØ induces cytokine responses, some of which parallel those observed by trauma patients' MØ (33). Consequently, we asked if IL-10 induced by FcγRI cross-linking stimulation of MØ followed by MDP stimulation could also be down-regulated by exogenous TGF-β addition, thereby testing the TGF-β down-regulatory effect on IL-10 in a more intensely stimulated MØ. Addition of 4.8 ng/ml of TGF-β would decrease the IL-10 induced by MDP stimulation subsequent to FcγRI cross-linking, as can be seen in the five representative experiments in Fig. 5. These data imply that patients' MØ IL-10 production could be suppressed by concomitant induction of TGF-β. Although trauma patients' MØ with elevated TGF-β levels always exhibited depressed IL-10 induction, some patients' MØ had depressed IL-10 induction capacity but no elevated TGF-β levels (data not shown). These data suggest that although postinjury hyperelevation of TGF-β levels can contribute to depressed MØ IL-10 levels in the posttrauma patient, they cannot be the sole explanation for the decreased MØ IL-10 production in immunosuppressed trauma patients.

DISCUSSION

Although there are no reports on posttrauma IL-10 levels, two recent reports suggesting that IL-10 levels were increased after hemorrhage, and another report of increased plasma levels of IL-10 in septic patients, would seem to imply that elevated IL-10 levels could be mediating the severe immunosuppression occurring in trauma patients (17, 18, 40). These data are difficult to reconcile with the highly elevated TNF-α and IL-6 production that is simultaneously occurring in many of these immunosuppressed patients and is directly associated with the posttrauma organ failure and mortality that occurs one to three weeks after the injury (2–7). IL-10 has been shown to suppress both MØ and PMN production of inflammatory cytokines (22–25). The data reporting elevated IL-10 after sepsis is also in contrast to a number of *in vivo* reports showing that animals depleted of IL-10 have dramatically increased susceptibility to endotoxin shock mortality and that administration of IL-10 can significantly improve survival during endotoxin shock by depressing TNF-α levels (14–16, 45). Other murine studies have shown that IL-10 protects from lung injury and inhibits MØ procoagulant activity, thereby decreasing lung failure due to disseminated intravascular coagulation (DIC) (46, 47). DIC is a common posttrauma sequel and would not be expected to occur concomitant with elevated IL-10 levels, again suggesting that IL-10 levels should be suppressed after trauma.

The data reported here may reconcile some of these seemingly disparate reports. Our trauma patients were separated into three groups based on their T-cell proliferative responses in comparison to normal controls. One group had both depressed mitogen responses in the

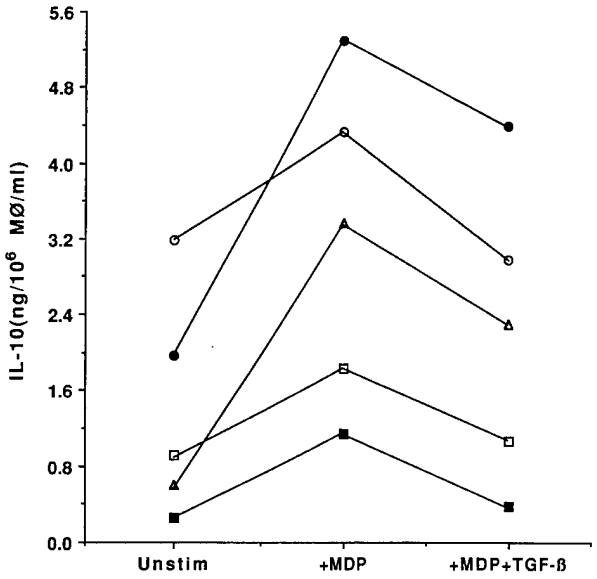

Fig. 4. Data of five representative experiments from 13 experiments, showing down-regulation of MDP induced MØ IL-10 production by TGF-β (P value for MDP vs MDP + TGF-β = 0.0003 for all experiments, as assessed by Wilcoxon nonparametric test). MØ from normals were cultured (3×10^6 cells/3 ml) for 16 hr in medium alone (unstimulated), MDP (20 μg/ml), or MDP (20 μg/ml) in presence of TGF-β (2.4 ng/ml) and the IL-10 levels in the culture supernates were measured by ELISA.

PBMC population and depressed T-cell proliferation in a MØ-independent proliferation system (anti-CD3 and anti-CD4 stimulation). The second patient group had depressed mitogen responses in the PBMC population, but normal or slightly elevated T-cell proliferation. The third patient group had elevated mitogen responses and significantly elevated T-cell proliferation. The IL-10 levels of T cells from these three groups of patients were dramatically different, while MØ IL-10 levels in both the mitogen-suppressed groups were depressed. Patients with depressed mitogen responses and depressed T-lymphocyte proliferation had both significantly depressed T-cell and MØ IL-10. In contrast, patients with depressed mitogen responses but normal or elevated T-cell proliferation capacity produced normal or elevated levels of T-cell IL-10 in response to anti-CD3, anti-CD4 stimulation, but had depressed MØ IL-10 levels. Patients with elevated mitogen responses had both highly elevated T-cell proliferation and highly elevated T cell IL-10 levels. MØ from patients with normal PBMC proliferation in response to mitogen also had normal IL-10 production.

The posttrauma appearance of a depressed PBMC mitogen response in the face of competent T-lymphocyte proliferation is explainable by suppressed T-cell induction to PHA in the PBMC population, secondary to increased MØ PGE_2 and MØ TGF-β production, as well as reduced T-cell numbers in the PBMC population (6–8, 37). Since MØ are the primary producers of IL-10 in the mitogen-stimulated PBMC population, depressed MØ IL-10 production makes these patients' PBMC IL-10 levels appear depressed when compared to normals' (25, 26). However, these patients had elevated T-cell IL-10 responses induced by anti-CD3, anti-CD4 if their T-cell proliferation was normal or elevated. It has been demonstrated that exogenous IL-10 can directly inhibit human T-cell APC-independent proliferation in response to anti-CD3 stimulation (28). Consequently, the elevated IL-10 levels of our patients' T cells could not have been mediating their depressed PBMC mitogen responses since their APC-independent T-cell proliferation was still normal or elevated and their PBMC IL-10 levels were depressed.

The reported data showing elevated plasma levels of IL-10 in septic patients is compatible with our data showing highly elevated T-cell IL-10 levels in many trauma patients with normal or elevated T-cell proliferation responses. Since many trauma patients exhibit an elevated level of T-cell proliferation (possibly in response to infectious challenge), a detection of elevated plasma IL-10 might be explained. However, trauma

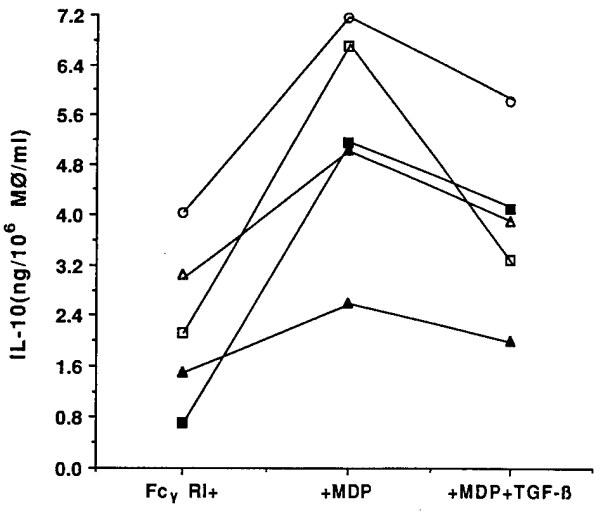

Fig. 5. Data of five representative experiments from 17 experiments, showing down-regulation of FcγRI cross-linked MDP-induced MØ IL-10 production by TGF-β (P value for MDP vs MDP + TGF-β = 0.0006 for all experiments, as assessed by Wilcoxon nonparametric test). MØ from normals, separated by rosetting with anti-Rh coated erythrocytes were cultured (3×10^6 cells/3 ml) for 16 hr in medium alone (FcγRI$^+$), MDP (20 μg/ml), or MDP (20 μg/ml) in presence of TGFβ (4.8 ng/ml), and the IL-10 levels in the culture supernates were measured by ELISA.

patients with elevated IL-10 resolved their infection and appeared to have normal immune responses. It was the trauma patients showing significantly decreased T-cell proliferation and depressed MØ and T-cell IL-10 production who went on to die, again suggesting that elevated IL-10 is not the mediator of trauma patients' immunosuppression. The data showing increased IL-10 production after hemorrhage in the murine systems may reflect known species differences, time of measurement differences, or a difference in hemorrhage alone versus multiple trauma. In one murine experiment, hemorrhage led to a three-day posthemorrhage increase in IL-10 production by isolated murine splenic $CD4^+$ T cells, *in vitro* stimulated with Con A, but no increase in IL-10 production by the whole posthemorrhage splenocyte population (17). Since there was no measurable IL-10 production in the total splenocyte population of either control or hemorrhage animals, elevated IL-10 levels could not have been affecting the total splenocyte proliferation responses. These hemorrhage model data showed increased IL-10 only in the isolated $CD4^+$ splenic cells.

Our data also include patients whose isolated T-cell IL-10 responses were increased when their total IL-10 levels in stimulated PBMC were depressed as compared to normals, but these patients had normal or elevated T-cell proliferation. Many of our patients initially appear to have had normal IL-10 levels, followed by a period of increased T-cell proliferation and increased T-cell IL-10 production, which then either progressed to severe immune suppression and decreased IL-10 levels or returned to a normal proliferation profile. The data are particularly intriguing because they may indicate an early selective increase in T-cell IL-10 production capacity detected by *in vitro* stimulation of the $CD4^+$ population. Patients with severe trauma show increasing T-cell depletion over time after injury (10). This depletion appears to result from T-cell apoptosis (48, 49). It might, therefore, be suggested that Th1 lymphocytes from trauma patients are more sensitive to posttrauma apoptosis as are Th1 lymphocytes from HIV patients (50). This would result in an initial increase in the proportion of Th2 lymphocytes that could result in the isolated $CD4^+$ population producing increased IL-10 while maintaining a normal or even elevated response to *in vitro* stimulation by anti-CD3, anti-CD4. However, such a Th1 depletion would need to be quickly followed by development of a total T-cell dysfunction with no T-cell proliferation and no IL-10 production to accommodate our immunosuppressed trauma patient data.

The early posttrauma loss of MØ IL-10 production could reflect such an early transient increase in T-cell IL-10, which then depresses MØ IL-10 levels. IL-10 depression of further MØ IL-10 production has been previously described (22). Excessive MØ TGF-β production could then also contribute to maintaining MØ IL-10 depression since elevated MØ TGF-β usually arises later in the postinjury period (37). During such an initial postinjury period of increased IL-10 production, inflammatory cytokine production would be appropriately controlled. If the T-cell dysfunction intensifies to include both Th1 and Th2 lymphocytes, as indicated by a failure of anti-CD3 and anti-CD4 to induce proliferation, then IL-10 levels are totally depressed and MØ and PMN production of inflammatory cytokines, such as TNF-α and IL-6, can proceed unabated. This model would incorporate our data showing depressed MØ and T-cell IL-10 production concomitant to increased MØ TNF-α levels, the *in vivo* murine data illustrating the protective effect of IL-10 against cytokine shock, and the recent murine hemorrhage model reports of early increased IL-10 levels. The hyperelevated MØ TNF-α levels seen in trauma patients more than seven days after injury were occurring concomitant with MØ inability to be induced for IL-10 production. Our data suggest that the severe posttrauma immunosuppression that occurs concomitant with hyperelevated cytokine levels is characterized by a loss of regulatory IL-10 production by both the MØ and T-cell populations. This loss of IL-10 activity may contribute to the overproduction of inflammatory cytokines, which is presumed to lead to patient mortality. In addition, IL-10 is important to B-cell maturation and has been reported to protect T cells from apoptosis (51, 52). Loss of IL-10 activity may, therefore, also allow increased T-cell apoptosis in the chronically stimulated T cells of trauma patients. A very early postinjury elevation in T-cell IL-10 levels may, therefore, represent a normal and appropriate response designed to temper the trauma-mediated activation of MØ, T cells, PMN, and B cells and prevent T-cell depletion. It is apparent, however, from the data presented here, that the severe T-cell immunosuppression that is associated with increased posttrauma mortality is not a result of elevated T-cell or MØ IL-10 production. Our data also suggest that the loss of MØ IL-10 production may be detrimental to the patients' regulation of inflammatory cytokines and may contribute to increased occurrences of end organ failure.

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A MEMBRANE TNF-α/TNFR RATIO CORRELATES TO MODS SCORE AND MORTALITY

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ABSTRACT—This study hypothesizes that post-trauma elevated membrane-associated tumor necrosis factor-α (mTNF) and decreased TNF receptor shedding may be more related to development of multiple organ dysfunction syndrome (MODS) than elevated secreted TNF-α. We also address several of the possible reasons for the previous conflicting reports in studies correlating trauma patients' sera TNF-α levels to their clinical outcome. These are 1) the lack of an objective quantitative score of clinical illness severity, 2) the lack of multiple TNF-α measurements in one patient to allow for trend analysis, 3) the lack of analysis of membrane-associated as well as secreted TNF-α levels, 4) the lack of concomitant analysis of soluble TNF-α receptors which may bind TNF-α in the serum, and 5) the possible requirement for more than one dysfunction in monocyte (Mϕ) TNF-α production and regulation to initiate pathology. Here, the MODS score was used to quantitate patients' illness severity over the length of their intensive care unit (ICU) stay. Patients' and normals' monocytes (stimulated and unstimulated) were assessed for production of secreted as well as membrane-associated TNF-α (sTNF and mTNF) and for shed p75 TNF-α receptor (TNFR) levels. These parameters of Mϕ TNF-α production and regulation were correlated to the MODS score as an indicator of clinical outcome. There was no correlation between sTNF and MODS score (p = .9025). There was a correlation between increased mTNF (p = .057) or decreased TNFR shedding (p = .0021) to increased MODS, but this lacked specificity. However, when the stimulated Mϕ production of mTNF and TNFR are expressed as the mTNF/TNFR ratio, an increased ratio correlates with high specificity to development of organ failure (p = .0002). These data indicate that a dual deregulation in Mϕ TNF-α production reflects increasing mTNF-α levels concomitant to decreased Mϕ shedding of neutralizing TNFR and correlates with the development of MODS.

INTRODUCTION

Tumor necrosis factor-α (TNF-α) has been repeatedly implicated as a mediator of multiple organ dysfunction syndrome (MODS) secondary to systemic inflammatory response syndrome (SIRS) after trauma and/or sepsis (1–4). However, it has been difficult to directly correlate levels of serum TNF-α with the onset of sepsis or MODS in patient populations (4–6). Some investigators even suggest that TNF-α levels are irrelevant in the pathogenesis of MODS (4). There are several possible reasons for this apparent discrepancy. The first may be a problem of objectively quantifying patient's clinical outcome so that it can be correlated to aberrant mediator function. Although mortality is frequently used as an endpoint, it is an all-or-none phenomenon and does not account for the pathological effects of early and/or nonfatal MODS caused by TNF-α. Previous investigators have suggested that mortality is an insensitive endpoint and that secondary endpoints, such as organ failure, ought to be used (7). Using incidence of "sepsis" as a marker for SIRS is fraught with difficulty in interpreting and defining sepsis and septic episode. It does not offer a grading scale to quantitate patient illness severity. The use of a clinical scoring system, such as Marshall's multiple organ dysfunction syndrome score (MODS score), facilitates objective quantitation of patients' physiologic status as a measure of the pathological effects of TNF-α (8).

The second problem in correlating TNF-α elevation as a cause of MODS may be one of timing. Often, TNF-α levels are measured upon admission or after onset of MODS. In the case of the injured patient, a post-trauma trend of increasing TNF-α production may be more predictive of outcome than the TNF-α level at an initial, single time point. In the case of MODS, the pathological high concentration levels of TNF-α production may precede, not coincide, with onset of organ dysfunction.

A third problem affecting the correlation of TNF-α to development of MODS may be the source of the TNF-α sample tested. Often, TNF-α levels are measured in the sera of patients. Since many cytokines are produced "locally" and act locally, the measured level of circulating cytokine may be poorly reflective of actual cytokine levels both because of low released levels in the circulation and because of the presence of circulating soluble receptors that bind TNF-α. In addition, measurement of sera levels of cytokine does not delineate the producing cell source nor identify cytokines that are cell associated. A number of cytokines, including TNF-α, have potent biologic activity while expressed on the producing cell surface (9–12). Although circulating monocytes (Mϕ) are most often implicated as the source of TNF-α, Mϕ at local sites may be equally or more important (13, 14). Cell-associated Mϕ TNF-α has been shown to be more cytopathic than secreted TNF-α for normal cells (10–12). Such cell-associated TNF-α would be

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more efficient in organ-specific damage because only Mϕ that were bound to the organ tissue through their up-regulated adhesion molecules or concentrated in the organ through directed migration would be mediating damage.

An additional problem with measuring serum TNF-α levels is the masking effect resulting from the presence of soluble TNF-α receptors (TNFR) that are able to neutralize secreted TNF-α and thus decrease the accuracy of serum measurements. Although there are two types of TNF receptors, the p55 and p75 types, the p75 is the primary Mϕ TNFR (15, 16). It has been previously shown that increasing levels of circulating p55 TNFR correlate with mortality in trauma patients (17, 18). The circulating p75 kDa receptor has also been shown as elevated post-injury, but the correlation to mortality has been less demonstrable (19). The ability of both anti-TNF-α antibodies and soluble TNF-α receptors to block cytokine shock in patient and animal models even when circulating TNF-α levels are not detected further suggests that cell-associated TNF-α is important in post-trauma sepsis and MODS and that circulating TNFR may be contributing to TNF-α neutralization (1, 2). Circulating TNFRs would interfere with the ability to detect serum TNF-α and thus would hinder the correlation to patient status while neutralizing circulating secreted TNF-α (sTNF). Cell-associated TNF-α would not be detected in the serum but would be susceptible to anti-TNF-α therapy.

To overcome the above problems with correlating TNF-α to clinical status in patients, the MODS score was used to objectively quantitate patients' clinical severity (8). We then hypothesized that a dual dysregulation of TNF-α production is required to lead to MODS. Specifically, we hypothesized that an increase in the more cytopathic mTNF concomitant to a decreased ability to mitigate mTNF's effects by shedding TNFR must occur to result in MODS. Multiple aspects of Mϕ TNF-α production were simultaneously assayed: the level of sTNF, the levels of cell-associated TNF-α (hereafter referred to as membrane TNF-α or mTNF) and the levels of shed p75 TNFR. The level of mTNF to TNFR was expressed as the ratio mTNF/TNFR to represent a simultaneous dysregulation of two controlling aspects of TNF-α production. Blood was collected twice a week from patients in order to establish a trend in TNF-α production.

MATERIALS AND METHODS

Patients

All studies were performed under protocols approved by the Institutional Review Board of the University of Massachusetts Medical Center, Worcester, MA. Informed consent was obtained from all healthy volunteers and from either patients or their next of kin. Twenty-five burn and trauma patients with an estimated injury severity score (ISS) greater than 15 and admission to the ICU were enrolled. There were 11 burn patients and 14 trauma patients, all with blunt trauma (71% motor vehicle accident) (Table 1). The ISS ranged from 13 to 59 with a median of 29. The ages of the patients ranged from 20 to 83, with a median of 39. One hundred and seven samples were tested on the 25 patients. A sample from one of our 31 normal volunteers was always processed in tandem with the patient samples. Ages of the normals ranged from 18 to 60. Blood was collected in a sterile manner within two days of admission and twice weekly until discharge from the ICU. Thirty milliliters was collected at each time and placed into heparinized tubes.

Calculation of MODS Score

On each day that blood was drawn, the patients' physiologic parameters for that day were collected. The value for the highest organ dysfunction occurring during the previous 24 h of this day for each of six organ systems was used to calculate the score as described by Marshall (8). The organ systems assessed were as follows: respiratory (Po_2/Fio_2), cardiovascular (heart rate $\times$ central venous pressure/mean arterial pressure) hematologic (platelet count), renal (creatinine), hepatic (bilirubin), and neurologic (Glasgow Coma Scale). Each organ system is scored 0 (no dysfunction) to 4 (complete failure) for a total maximum score of 24. A score of 7 or greater represents at least two systems with severe dysfunction. Marshall found a score of >6.4 to be consistent with a sharp increase in mortality (8). We, therefore, grouped our patients based on a MODS score of either 0–6 or >6 in order to similarly define the high risk patients. We further subdivided the lower risk group into those with a score of 0–2, representing minimal dysfunction, and those with a score of 3–6, representing some organ dysfunction or no more than a single system failure to further distinguish subtle physiological dysfunction attributable to TNF-α dysregulation.

Temporal relationship between TNF dysregulation and worsening organ dysfunction

A subgroup (8 out of 25) of patients who met the two following criteria was identified: 1) they were followed consecutively for two or more weeks, and 2) their clinical condition worsened after admission, as determined by an increase in MODS score of at least two points. This excludes the patients with uncomplicated clinical courses and those who did not survive long enough to be studied at multiple points. The mean post-injury day for the muramyl dipeptide (MDP)-stimulated peak sTNF, peak membrane-associated TNF (mTNF), peak ratio, or nadir of TNFR shedding in these eight patients was calculated to determine temporal relationships between each of the aberrations. For each patient, the MODS scores were examined to determine the day that organ dysfunction was apparent or worsened (as determined by an increase of at least two points in MODS score).

Separation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) from normals' or patients' blood were isolated by centrifugation of heparinized blood over Ficoll-Hypaque gradient at 1600 rpm for 20 min. The cells were washed twice with HBSS and then suspended in RPMI 1640 supplemented with 8% screened serum, 50 U/mL penicillin-G, 50 μg/mL streptomycin, 50 μg/mL gentamycin, 2.5 μg/mL fungizone, 4 mM L-glutamine, 1 mM Na pyruvate, and 1% essential medium (MEM) nonessential amino acids. Endotoxin contamination was less than 15 pg/mL in the culture media and sera, and all media contained 100 U/mL of polymyxin B sulfate.

Isolation and stimulation of monocytes

Monocytes from patients' and normals' blood were separated from mononuclear cells by selective adherence as previously described (20). Briefly, 1–1.2 $\times$ 10^7 mononuclear cells in 12 mL of media supplemented with 18% FBS (Sigma Chemical Co., St. Louis, MO) are placed in 75 cm^2 culture flasks at 37°C (Corning Inc., Corning, NY). Following a 2 h adherence, nonadherent cells were removed. Adherent cells were removed by EDTA treatment and scraping. We have previously shown a >95% Mϕ purity by flow cytometric analysis (21). Normals' and patients' Mϕ are placed in 12-well plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) at 1.5 $\times$ 10^7 cells/mL in media with 15% FBS and cultured overnight either stimulated with 20 μg/mL MDP

TABLE 1. **Patient characteristics**

	Median ISS	Range	Median age (yr)	Range (yr)	Mortality
Trauma N = 14	40	22–59	34	20–76	7%
Burn N = 11	25	13–38	46	21–83	45%
Total N = 25	29	13–59	39	20–83	20%

or without further stimulation. MDP is a Gram-positive cell well analog. Because of the high levels of circulating endotoxin in trauma and burn patients, we use MDP instead of LPS to stimulate Mϕ that may have become tolerant to further LPS stimulation (22). Mϕ supernatants from stimulated and unstimulated cultures were collected after 16–18 h of stimulation and kept frozen at −80°C until TNF-α and TNFR were assayed. Adherent Mϕ from both unstimulated and stimulated cultures were also collected by EDTA treatment and scraping. Recovered cells were kept frozen until further analysis of membrane-associated TNF-α.

TNF-α bioassay

TNF-α activity in Mϕ supernatants (secreted TNF-α) and sonicated Mϕ lysates (mTNF) were measured in the L-M cell bioassay as previously described (20, 23). Values are expressed as pg/10^6 cells/mL. Some samples were also assayed for secreted TNF-α using a TNF-α ELISA (Medgenix Diagnostics, Belgium) which detects both bound and free TNF-α. We have previously shown that the cell-associated TNF-α activity is completely neutralized by anti-TNF-α antibody.

TNFR assay

p75 TNF-α receptor in Mϕ supernatants was measured by ELISA (Medgenix) according to manufacturer's instructions. This ELISA detects both free TNFR and TNFR bound to TNF-α.

Calculation of mTNF/TNFR ratio

The values for the TNFR from both stimulated and unstimulated Mϕ cultures were first multiplied by 100 to allow for logarithmic analysis and plotting of values <1. Then the mTNF value (Mϕ membrane lysates TNF-α assay) were divided by the TNFR value (obtained from the supernatant) from the same time point and culture for each patient and each time point. The peak ratio attained by the patient over their clinical course was then represented.

Statistics

Chi-squared with Yate's correction factor (Microstat) is used to evaluate mortality and MODS ≤6 or >6. Kruskal-Wallis one-way ANOVA (BMDP) was used to evaluate differences between MODS groups of 0–2, 3–6, vs. >6. Statistical significance was set at $p < .05$.

RESULTS

Correlation of MODS score to Mϕ TNF-α parameters

The patients were grouped according to their MODS score (0–2, 3–6, or >6) on the day that a peak sTNF, peak mTNF, peak ratio, or nadir of TNFR shedding was reached, as represented in Fig. 1 and all subsequent figures. The MODS score represented for the Mϕ TNF-α parameter does not necessarily represent the maximal MODS score for each patient.

Examination of Mϕ-secreted TNF-α levels

Since most trauma patient studies have focused on serum TNF-α levels, we first examined the levels of secreted TNF-α in Mϕ culture supernatants (24–29). It was reasoned that since Mϕ are purported to be the primary producers of TNF-α, their secreted TNF-α levels should be reflective of serum levels (30). Matrix isolated Mϕ were cultured alone or in the presence of MDP, and the highest level of sTNF reached in a patient was compared with their MODS score on that day (Fig. 1 and Table 2). Most patients' Mϕ had undetectable or very low levels of sTNF throughout their course. However, those patients' Mϕ that did secrete significant levels of sTNF did so early on in their clinical course (peak sTNF in first 4 days post-injury). This peak level of Mϕ-secreted TNF-α was compared with the patient's concomitant MODS even though a higher MODS might be reached on subsequent post-injury days. There is no correlation between sTNF and MODS score

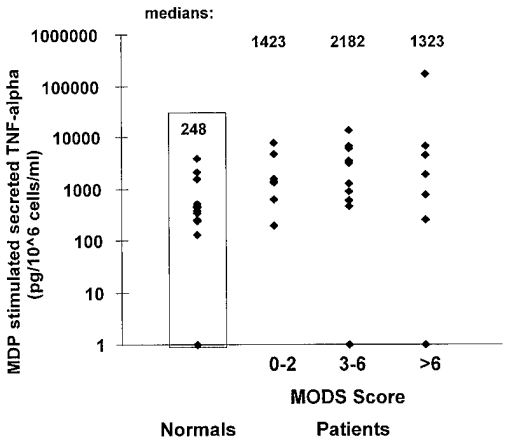

FIG. 1. **Mϕ-secreted TNF-α does not correlate to severity of organ dysfunction.** Matrix-isolated Mϕ from normals and patients were cultured and stimulated 16–18 h with MDP (20 μg/mL), and then supernatants were collected. Levels of biologically active secreted TNF-α (sTNF) in the supernatants were assayed in the L-M cell bioassay. The peak level of sTNF produced by a patient during their study period is represented along with the multiple organ dysfunction syndrome (MODS) score on that day. Analysis of variance between low (0–2), medium (3–6), and high (>6) MODS score groups showed no correlation between sTNF and MODS ($p = .9025$).

at any time. All patients' Mϕ, as a group, had a higher level of sTNF than the normals' Mϕ regardless of MODS or post-injury day. ELISA results were usually slightly higher than biological results, reflecting some receptor bound TNF-α, but ELISA results exactly paralleled biological assay results in that when no sTNF was detected by biological assay, little or no sTNF was detected by ELISA (data not shown).

When the highest level of sTNF from unstimulated patients' Mϕ is compared with their MODS score on that day, there is no correlation (ANOVA $p = .5566$ for MODS score grouped by low, medium, or high). Even when the patients' Mϕ are stimulated with MDP to simulate the increased TNF-α production by preactivated Mϕ to a secondary bacterial challenge, there is still no correlation between levels of sTNF and MODS score. (ANOVA $p = .9025$) (see Fig. 1 and Table 2). All 25 patients are represented once in Fig. 1 by their highest Mϕ sTNF level. These data confirm other studies on serum TNF-α levels and demonstrate that peak sTNF levels do not appear to correlate with development of MODS (31). It should be noted that MDP-stimulated Mϕ from three patients in the moderate to high MODS score group never demonstrated any sTNF by L-M bioassay or by ELISA.

Examination of membrane-associated TNF-α levels

We have previously found that patients might have little or no sTNF but may instead have high levels of membrane-associated TNF-α (mTNF) and that mTNF levels appeared to more closely parallel patient outcome (21, 32). A number of other investigators have also shown that mTNF correlates with organ damage in both patient and animal trauma models (14, 33). We, therefore, simultaneously examined the levels of mTNF on the patients' Mϕ as well as their secreted TNF on multiple post-injury days. Mϕ from normals and patients were cultured either alone or stimulated with MDP and the cell

TABLE 2. **Summary of sTNF, mTNF, TNFR, and ratio versus MODS score in the total patient population**

TNF-α variable	Cutoff	Normals	MODS 0–6	MODS > 6	ANOVA	χ^2 MODS	χ^2 mortality
sTNF (pg/10^6 cells/mL)		(median)	(median)	(median)			
UNS	N/A	0	109	0	$p = .5566$	ND	ND
MDP (Fig. 1)	N/A	248	1,423	1,323	$p = .9025$	ND	ND
mTNF (pg/10^6 cells/mL)		(above/below cut-off)	(above/below cut-off)	(above/below cut-off)			
UNS	10,000	0/25	5/11	8/1	$p = .0280$	$p = .0187$	$p = .0572$
MDP (Fig. 2)	10,000	0/25	8/11	6/0	$p = .0567$	$p = .0435$	$p = .0868$
TNFR (ng/10^6 cells/mL)		(below/above cut-off)	(below/above cut-off)	(below/above cut-off)			
UNS	.5	0/25	2/12	3/8	$p = .0193$	$p = .762$	$p = .0868$
MDP (Fig. 3)	2.0	0/25	2/12	9/2	$p = .0021$	$p = .003$	$p = .0356$
mTNF/TNFR ratio		(above/below cut-off)	(above/below cut-off)	(above/below cut-off)			
UNS	100	0/25	5/10	8/2	$p = .0062$	$p = .118$	$p = .4808$
MDP (Fig. 4)	100	0/25	1/15	8/1	$p = .0005$	$p = .0002$	$p = .0766$

membrane lysates assayed for mTNF in the L-M cell bioassay. The peak level of Mϕ mTNF attained by a patient was selected and compared with the MODS score on that peak day. The peak post-injury day of mTNF for patients who developed severe MODS occurred later than the peak of their sTNF. In fact, Mϕ sTNF levels were usually low or absent in these patients' Mϕ at this peak mTNF period. The peak level of mTNF on unstimulated patients' Mϕ correlated to their MODS score on that day (ANOVA $p = .0280$). However, there are several false positives (i.e., patients with high levels of mTNF and low MODS score), and there is a weak correlation to mortality (chi-squared $p = .0572$) (Table 2). Furthermore, when the same patients' Mϕ are stimulated with MDP and their peak levels of mTNF examined, the correlation to clinical outcome as represented by the MODS score on that peak day is minimized (ANOVA $p = .0567$) (Fig. 2, Table 2). Again, the peak of Mϕ mTNF occurred later than the peak of sTNF (post-injury day 8; Table 3). If a cutoff of 10,000 pg/10^6 Mϕ/mL of mTNF is established post-hoc, all of the normals' Mϕ produce less than that mTNF concentration and all of the patients with MODS score >6 produced greater amounts while specificity is maximized and no false negatives occur. Yet, again, there are a number of false positives (eight patients) (Table 2). Finally, when mortality is examined, there is no correlation to peak mTNF levels (chi-squared $p = .0868$).

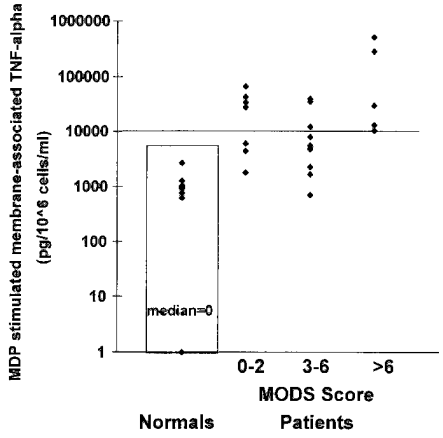

FIG. 2. **Mϕ membrane-associated TNF-α correlates to organ dysfunction but lacks specificity.** Matrix-isolated Mϕ from normals and patients were cultured 16–18 h with MDP. Mϕ membranes were assayed for levels of membrane-associated TNF-α (mTNF) in the L-M cell bioassay. Both the peak mTNF level produced during the patients' study period and the MODS score on that day is presented. Analysis of variance between low (0–2), medium (3–6), and high (>6) MODS score groups and mTNF (ANOVA $p = .0567$) and chi-squared analysis using a cut-off of 10,000 pg/10^6 Mϕ/mL mTNF and MODS score ≤ 6 or >6 is employed (chi-squared $p = .0435$).

Examination of shed p75 TNF-α receptors

Increased TNF-α production has been shown to parallel increased TNFR production at both the protein and the molecular level suggesting that TNF-α production and TNFR neutralization are tightly linked (34, 35). Our laboratory has previously shown increased TNFR on the surface of Mϕ in trauma or burn patients who also experienced an increase in Mϕ mTNF (36). These data imply that shedding of the Mϕ TNFR (p75) might be defective in some patients. Other investigators have noted an increase in p55 TNFR associated with pathology. Mϕ, however, produce mainly the 75 kDa form of the TNFR, and shedding of this 75 kDa receptor correlates poorly to MODS or mortality (16, 34). Recent data suggest that the p75 TNFR may preferentially bind mTNF as well as sTNF, suggesting that the shed p75 receptor may be critically important in neutralizing mTNF on Mϕ infiltrating target organ sites (12). We, therefore, examined the levels of p75 TNFR shed by the matrix isolated, unstimulated and stimulated, normals' and patients' Mϕ simultaneous with their sTNF and mTNF levels. When the unstimulated patients' Mϕ supernatants were examined for their levels of shed TNFR, many patients' Mϕ actually showed decreased levels of TNFR in their Mϕ supernatants, supporting a defect in TNFR shedding. A correlation was noted between the nadir of Mϕ TNFR shedding and an increased MODS score (ANOVA MODS score grouped 0–2, 3–6, >6 $p = .0193$). If a cutoff of .5 ng/10^6 Mϕ/mL of TNFR is established, then all the normals' Mϕ will shed higher levels and most of the patients' Mϕ with corresponding MODS score >6 will produce lower levels. There are then two false positives (low TNFR and low MODS) and no correlation to mortality (chi-squared $p = .0868$) (Table 2). More significantly, there is now a problem with false negatives (three patients).

When the TNFR shedding in MDP-stimulated patients' Mϕ

TABLE 3. **Temporal relationship of peak sTNF-α, mTNF-α, ratio, and TNFR nadir to increasing MODS**

	Mean PID Peak				PID
	sTNF	mTNF	TNFR	Ratio	MODS ↑
Trauma (n = 3)	x	10	13.3	10	11
Burn (n = 5)	x	6.8	10.8	11.7	10
Total (n = 8)	x	8	11.75	11.4	10.4

A subgroup of eight patients was identified who were followed two or more weeks and whose MODS scores worsened from the time of admission (>2 point increase). The mean post-injury day (PID) for the sTNF peak, mTNF peak, ratio peak, or the nadir of TNFR shedding is presented. For each patient, the MODS scores over time were analyzed to determine the time post-injury when MODS worsened (2 points).
x, No significant secretion.

was examined, the correlation to MODS score and mortality was improved (ANOVA $p = .0021$, mortality chi-squared $p = .0356$) (Fig. 3, Table 2). If a cutoff of 2.0 ng/10^6 Mϕ/mL of TNFR is established, then all of the normals' Mϕ will shed higher levels and most of the patients' Mϕ with corresponding MODS score >6 will produce lower levels. However, specificity continues to be a problem and now sensitivity is impaired with two false negatives occurring. In the eight patients with worsening MODS, their nadir of TNFR shedding occurred on average 11.75 days post-injury (Table 3).

Examination of mTNF/TNFR ratio

The above Mϕ data demonstrate that both increased mTNF levels and decreased TNFR shedding either correlate or come close to showing a correlation with MODS score but that both of these parameters either lack specificity, since there is a high false positive rate, or lack sensitivity (false negatives). It was hypothesized that since both increased levels of Mϕ mTNF and decreased levels of shed Mϕ TNFR are involved in elevated TNF-α activity, the correlation to organ dysfunction might be strengthened if both of these aspects of TNF-α production could be evaluated together. It can be reasoned that increased cell-associated TNF-α by itself would not necessarily be destructive as long as there was an appropriate increase in p75 TNFR shedding to neutralize its effects. Thus, an increased Mϕ mTNF and concomitant failure to shed TNFR should more strongly correlate to worsening organ failure. To incorporate both these two aspects of Mϕ TNF-α activity, the patient data are expressed as their mTNF/TNFR ratio. In this analysis, the higher the ratio, the worse the dysregulation of TNF-α production.

When the peak of the mTNF/TNFR ratio for unstimulated patient Mϕ is analyzed, there is a statistical difference between the patient groups with MODS score >6 versus those with MODS score ≤6 (ANOVA $p = .0062$). If a ratio of 100 or greater is established post-hoc as a cutoff, all the normals' Mϕ ratios are far below this cutoff. Most patients (10/15) with MODS score ≤6 also have Mϕ mTNF/TNFR ratios <100 (Table 2). Finally, all but two patients with MODS >6 have mTNF/TNFR ratios well above 100. When using this ratio of mTNF/TNFR from unstimulated Mϕ, a problem with specificity still remains. This is illustrated by chi-squared analysis of patients with a mTNF/TNFR ratio above or below 100 and MODS score above or below 6, showing no correlation ($p = .1181$) (Table 2).

The problem with specificity is minimized when the mTNF/TNFR ratio of MDP-stimulated Mϕ is examined (Fig. 4, Table 2). There is now a very strong statistical difference between increased MODS score and a patient's peak ratio (ANOVA MODS score grouped 0–2, 3–6, >6 $p = .0005$). When considering patients with a MDP-stimulated peak Mϕ mTNF/TNFR ratio of 100 or greater as the high risk patients, there is only one false positive (mTNF/TNFR ratio >100 but MODS score ≤6) and only one false negative (ratio <100 but MODS score >6). No sample was obtained from the false negative patient during the critical day 10 to day 20 period, raising the possibility that a ratio >100 occurred but was undetected in

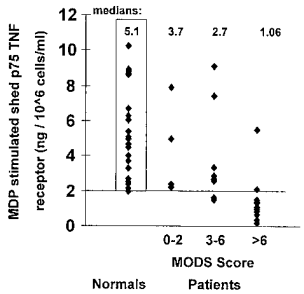

FIG. 3. **Mϕ shed p75 TNF-α receptor strongly correlates to organ dysfunction but has suboptimal specificity.** Levels of shed p75 TNFR in supernatants of patients' or normals' Mϕ stimulated with 20 μg/mL MDP were assayed by ELISA. The nadir of the patient's p75 TNFR during their study period is represented along with the MODS score on that day. There is a significant difference between low (0–2), medium (3–6), and high (>6) MODS score groups ($p = .0021$). Chi-squared analysis using a cut-off of 2.0ng/10^6 Mϕ/mL and MODS score ≤6 or >6 was significant at $p = .003$.

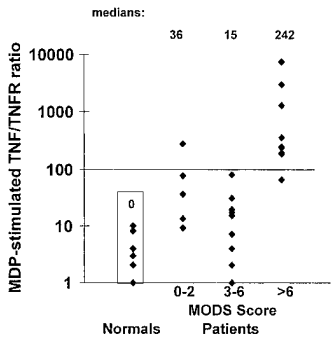

FIG. 4. **Mϕ mTNF/TNFR ratio strongly correlates to organ dysfunction with high specificity.** For each time point that patients' Mϕ were studied, a mTNF/TNFR ratio was calculated. The patients' peak Mϕ mTNF/TNFR ratio during their study period is represented along with the MODS score on that day. There is a highly significant difference ($p = .0005$) between low, medium, and high MODS score groups. Chi-squared analysis using a cut-off ratio of 100 and MODS ≤6 or >6 is significant at $p = .0002$.

this patient. All of the normals were well below the cutoff of a ratio = 100 and the median normal stimulated Mϕ value was a mTNF/TNFR ratio = 0. In the eight patients with worsening organ failure, the mTNF/TNFR ratio on average peaked at post-injury day 11.4 (Table 3).

When the three MODS score (0–2, 3–6, >6) groups for the MDP-stimulated ratio were analyzed for differences in the ages of patients or differences in injury severity scores, there was no statistical significance (Figs. 5 and 6). Thus, older age or a more severe injury does not account for a higher MODS score or a higher mTNF/TNFR ratio. Identification of an elevated MDP-stimulated Mϕ mTNF/TNFR ratio appears to truly differentiate two patients of similar age and ISS: one patient who will suffer MODS subsequent to appearance of a high Mϕ TNF-α ratio and thus may benefit from specific therapy and the other patient who will have an uneventful recovery.

A chi-squared analysis of the MDP-stimulated ratios < or >100 versus mortality or MODS score ≤6 or >6 revealed a better correlation to MODS than to mortality (MODS score $p < .0005$ and mortality $p = .0215$). These data (Table 4) again illustrate that organ failure scoring is a much more sensitive marker for Mϕ TNF-α pathology than the gross outcome of mortality.

DISCUSSION

There has been some recent discussion of whether TNF-α plays any role in post-injury multiple organ failure (1–6). This question has arisen both because of difficulties correlating serum TNF-α levels to mortality and because TNF-α modulating therapy has proved unsuccessful in modifying post-trauma MODS (7). The clinical failure of TNF-α modulating therapy has been suggested as partially resulting from a failure to narrowly define an appropriate patient population and to correlate the treatment outcome with an appropriate endpoint. When mortality is used as an endpoint for correlating any immune inflammatory parameter to patient outcome, it does not allow for evaluation of nonlethal consequences of an immune inflammatory aberration (7). A less objective measure of patient outcome frequently used is incidence of infectious complications. However, this clinical correlation does not differentiate between a localized infection versus an infection with systemic sequelae. The multiple organ dysfunction syndrome score developed by Marshall offers an objective mechanism to evaluate the systemic sequelae of an infectious or

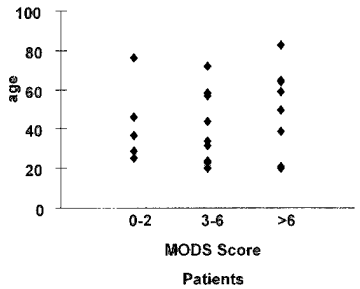

FIG. 5. **Injury severity score does not correlate to organ dysfunction.** The patient groups from Fig. 4 are represented here plotted against their injury severity scores (ISS). Analysis of variance between groups reveals no difference in ISS between groups.

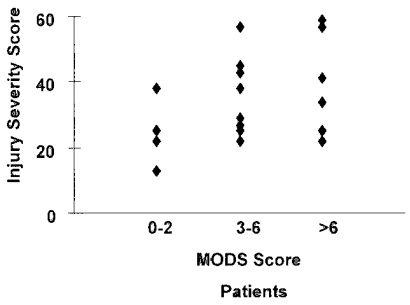

FIG. 6. **Age does not correlate to organ dysfunction.** The patient groups from Fig. 4 are represented here but are plotted against their ages. Analysis of variance between groups reveals no difference in age between groups.

TABLE 4. **Demonstration of higher specificity of mTNF/TNFR ratio to MODS score than to mortality**

mTNF/TNFR ratio (MDP)	MODS score		Mortality	
	≤6	>6	Alive	Dead
<100	15	1	15	1
>100	1	8	5	4

χ^2 MODS $p = .0002$; χ^2 mortality $p = .0766$.

septic episode or of an immune inflammatory aberration (8). We would propose that measurement of an increased mTNF/TNFR ratio represents a more sensitive indicator of aberrant TNF-α activity than assessment of sTNF. The enhanced ability of the MODS score compared with mortality to evaluate the consequences of TNF-α dysregulation illustrates the point that organ failure is also a more sensitive endpoint to use to establish clinical importance of a particular inflammatory aberration than mortality.

An ultimate goal might be to identify one parameter of TNF-α dysregulation that can be assayed and used for designing therapy or clinical trials. However, when evaluating peak mTNF for this function, there is high sensitivity and, therefore, all or most patients with organ failure would meet entry criteria based on elevated mTNF. However, costs would be increased because of entry of too many false positives. When evaluating decreasing shed TNFR or its nadir, there is the clinically more important problem of having false negatives. These occur in people who might potentially benefit from treatment but who would not be treated. Also, the nadir of shed TNFR occurs later than the increase in MODS score, thus decreasing the likelihood of being able to affect patient outcome.

When using MODS as our measure of clinical outcome, peak levels of Mϕ-secreted TNF-α did not correlate to patient outcome. These data may reflect the difficulty expressed in the literature of correlating serum levels of TNF-α to patient outcome. Our data further suggest that measuring serum levels of TNF-α that reflect sTNF levels only may be a poor means of evaluating trauma patient eligibility or timing of patient treatment in TNF-α modulatory therapy trials, such as those using anti-TNF-α antibody or soluble TNF receptor. Similarly, assessing alterations in serum TNF-α levels after anti-TNF-α therapy may be a poor marker for evaluating efficacy of TNF-α modulating therapy. The results of our study also support the hypothesis that mTNF is more cytopathic in MODS than sTNF

in that we found increased levels of mTNF were better correlated to organ failure than increased secreted TNF-α levels. mTNF has been suggested as capable of cytolysing normal human endothelial cells, whereas these cells are resistant to sTNF (12).

Although there was a stronger correlation of elevated patient Mϕ mTNF levels to development of multiple organ dysfunction, the correlation was just below statistical significance. These data suggested that more than one dysfunction in Mϕ TNF-α regulation might be required to result in organ pathology from TNF-α activity. Another regulatory function in controlling total *in vivo* TNF-α activity is the shedding of TNF receptors (15, 17–19). Two TNFR, one 75 kDa and the other 55 kDa, are produced by a variety of cell types and can be shed from the cell surface (35). Although both the 75 kDa and the 55 kDa soluble shed receptors can bind to secreted TNF-α, the 75 kDa type is the primary receptor on Mϕ and preferentially binds mTNF (9, 12). Therefore, its shedding would be more involved than the 55 kDa TNFR in localized neutralization of Mϕ mTNF (15, 16). In addition, the p75 receptor has been implicated as increasing the TNF-α cytotoxic activity for cells bearing the p55 receptor by TNF-α ligand passing (35). Although transfer and concentration of sTNF from the 75 kDa TNFR to the 55 kDa TNFR has only been demonstrated on the same cell, binding of secreted TNF-α back to the Mϕ receptors has been demonstrated (37). Consequently, transfer of sTNF bound onto the Mϕ 75 kDa TNF receptor to a 55 kDa receptor on target normal cells in the susceptible organs may represent another mechanism for increased TNF-α cytotoxicity. In addition, this laboratory has previously shown increased expression of TNFR on trauma patients' Mϕ associated with increased mTNF (36). Consequently, our data showing decreased Mϕ p75 TNFR shedding as correlated to increasing MODS score is consistent with data in the literature, suggesting that an imbalance of TNF-α and TNFR shedding is associated with increased mortality.

Although both the peak of elevated Mϕ mTNF and decreased Mϕ TNFR shedding each separately correlate to organ failure, the correlation and specificity of elevated Mϕ TNF-α activity aberrations and increasing MODS score can be enhanced by evaluating both aspects of TNF-α dysregulation in tandem. These data showing that a high ratio of mTNF to shed TNFR (indicating increased mTNF concomitant to decreased TNFR shedding) is correlated to MODS support a requirement for both an increased mTNF and failure to appropriately shed TNFR in order to cause organ failure. Taken together, the post-trauma increase in Mϕ mTNF and the failure to shed the 75 kDa receptor may greatly increase the potential of Mϕ infiltrating and adhering on lungs and on liver to cytotoxically destroy cells of normal organs by TNF-α-related mechanisms. These Mϕ expressing mTNF would preferentially bind to target cells expressing the p75 receptor lysing these cells that are resistant to sTNF. In addition, Mϕ failing to shed their p75R would not neutralize mTNF, but could bind sTNF to these receptors, concentrate it, and then pass these sTNF back to 55 kDa receptors on normal organ cells greatly increasing the lytic potential of sTNF (35). The close correlation of the altered mTNF/TNFR ratio to the development of MODS may be occurring primarily during the clinical course of burn and trauma patients. The strong correlation and high specificity of the mTNF/TNFR ratio in predicting MODS could make these parameters excellent endpoints for evaluating clinical trial efficacy or for determining patient entry criteria into a clinical trial whose goal is to manipulate TNF-α levels (i.e. anti-TNF antibody or sTNFR). It may be possible to use trend analysis to identify high risk patients early on by noting an increasing ratio rather than waiting for their ratio to reach its peak. It may also be possible to use specific antibody to rapidly assess both mTNF and TNFR levels on patients' Mϕ using flow cytometry as had been previously reported (23, 36). Such a rapid assay system would further facilitate use of the mTNF/TNFR ratio to evaluate trauma patient status. It has been suggested that distinguishing patients in whom TNF-α modulation would be beneficial from patients in whom elevated TNF-α levels are necessary for infectious disease control is a major clinical problem (38). Using the mTNF/TNFR ratio, it may also be possible to have a rapid and specific detection system of TNF-α dysregulation, thus enabling detection of patients who may benefit from anti-TNF-α therapy.

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Relationship of Post-trauma Altered Interleukin-12 and Interleukin-10 to Depressed Patient Mitogen Responses

A. K. De, K. Kodys, S. Fairfield, C. Miller-Graziano

Introduction

A number of investigators have demonstrated that severe mechanical or thermal trauma results in a decrease in the response of trauma patients' peripheral blood mononuclear cells (PBMC) to mitogen-induced proliferation [1-4]. Deficit in monocyte (MØ) antigen-presenting function, excessive MØ production of suppressive mediators such as prostaglandin (PG)E_2 and transforming growth factor ß (TGFß), or an actual T cell dysfunction have all been proposed as mediating this post-trauma loss of mitogen responsiveness [1-9]. In this series of experiments, trauma patients with injury severity scores > 35 or with > 30% 3° burns who also experienced depressed mitogen response were assessed for a deficit in T cell proliferation induced in a MØ-independent system. Those patients were concomitantly assessed for MØ production of interleukin (IL)-10, a potent inhibitor of T cell proliferation [10-13]. In addition, the ability of the newly identified "immune initiation cytokine", IL-12, to restore any trauma-induced T cell deficit was explored [14-16].

Examination of Post-injury T Cell Proliferation

Early examination of the effects of trauma on mitogen-induced proliferation resulted in publications claiming that trauma increased patients' mitogen responses [17, 18]. These conflicting reports resulted from two different data analyses problems. First, a highly increased nonspecific proliferation raised proliferation background levels of the patients' PBMC and, therefore, increased the apparent proliferation level of the patient. Second, averaging of all patient data, rather than examining individual patients' PBMC responses over time post-injury, led to combining patients who had increased PHA response and no negative clinical outcome with patients whose normal proliferation level was high and, consequently, a 50% proliferation decrease still placed their response levels at the proliferation values expressed by normal low responders [17, 18]. Examination of individual patient's mitogen responses properly corrected for nonspecific background proliferation produced

data such as that illustrated in Table 1 [1, 2]. Only those patients with long-term repeated episodes of sepsis showed depressed mitogen-induced proliferation. Trauma patients who properly responded to infectious challenge showed an elevated level of proliferation indicative of an ongoing normal T cell activation and no immune abnormalities. In contrast, trauma patients identified as having depressed mitogen responses also experienced a gamut of other immune dysfunctions including reduced IL-2 production, reduced IL-2 receptor (IL-2R) expression, increased PGE_2 production, decreased MØ antigen presenting cell (APC) capacity, and decreased NK cell activation [1-9, 19-21]. Studies focusing on the role of the monocyte as both a necessary costimulation of T cell proliferation and a source of inhibitors

Table 1: Correlation of burn patients' immunologic parameters to clinical course

	Maximum PHA Variation (%)	Outcome
Group I		
Patient		
1	+20	Non complications - released
2	-18	Non complications - released
3	-15	Non complications - released
4	+25	Non complications - released
5	+30	Non complications - released
6	+40	Non complications - released
7	-12	Non complications - released
Group II		
Patient		
1	+220	Pseudomonas infection - recovered
2	+260	Pseudomonas infection - recovered
3	+200	Deep vein thrombosis - recovered
4	+185	Staph, infection - recovered
5	+300	Staph, infection - recovered
6	+450	Pseudomonas infection - recovered
Group III		
Patient		
1	-70	Succumbed to Staph, sepsis
2	-80	Succumbed to Serratia, sepsis
3	-71	Succumbed to Pseudomonas
4	-80	Succumbed to Pseudomonas
5	-65	Succumbed to Enterococci
6	-85	Succumbed to Staph, sepsis
7	-72	Succumbed to Serratia, sepsis
8	-60	Pseudomonas, Staph-eventually recovered
9	-60	Staph, Serratia, Enterococcus, septicemia-eventually recovered

PHA, phytohemagglutinin

of T cell proliferation seemed to imply that much of the post-trauma depressed T cell proliferation could result from MØ dysfunction in T cell costimulation and/or MØ overproduction of inhibitors [22-26].

The question of the appearance of a post-trauma T cell deficit was confused by the use of proliferation systems in which contaminating MØ could influence the proliferation outcome. In this study, to examine patient T cell proliferation responses in the absence of MØ, the patients' PBMC were first depleted of cells adherent to microexudate-coated plastic (MØ), then the nonadherent cells were enriched for T cells by selecting the E rosette (neuraminidase-treated sheep erythrocytes) positive cells [27]. This highly T cell-enriched population no longer responded to stimulation by phytohemagglutinin (PHA), indicating MØ depletion. These purified T cells were then induced to proliferation by stimulation with anti-CD3 (1.5μg/ml) and anti-CD4 (1 μg/well) immobilized to plastic surface. This anti-CD4 and anti CD-3 stimulation regimen has been demonstrated to induce T cell proliferation in the absence of MØ and to preferentially induce T helper lymphocytes [27, 28]. Patients with depressed PHA responses were examined for a T cell proliferation dysfunction in this MØ-independent system. As can be seen in Figs. 1 and 2, patients with depressed PHA responses could have either normal levels of T lymphocyte proliferation in a MØ-free system (Fig. 1) or severely depressed levels of T cell proliferation concomitant to depressed PHA responses in the total PBMC population (Fig. 2). In fact, one patient could exhibit both a normal level of T cell

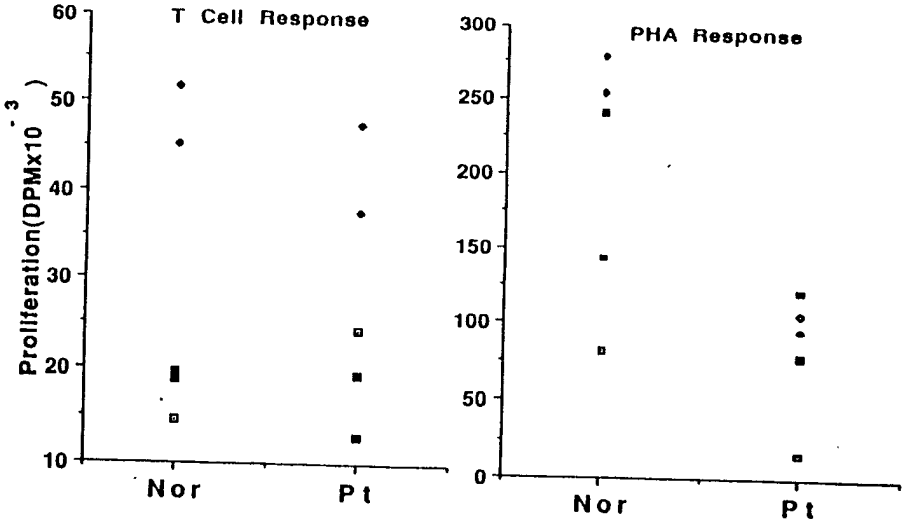

Fig. 1: Normal levels of T cell proliferation in monocyte (MØ)-free system in a group of trauma patients who have depressed ($p < 0.027$) phytohemagglutinin (PHA) responses. Normals (Nor) and patients (Pt) peripheral blood mononuclear cells (PBMC) (PHA response) and MØ-depleted E rosetted T cells (T cell response) ($2x10^5$ cells/200 μl/well) were assessed for proliferation (^{3}H-thymidine incorporation) at 72 h of culture in response to PHA and immobilized anti-CD3 (1.5 μg/well) + anti-CD4 (1 μg/well), respectively, and expressed as DPM.

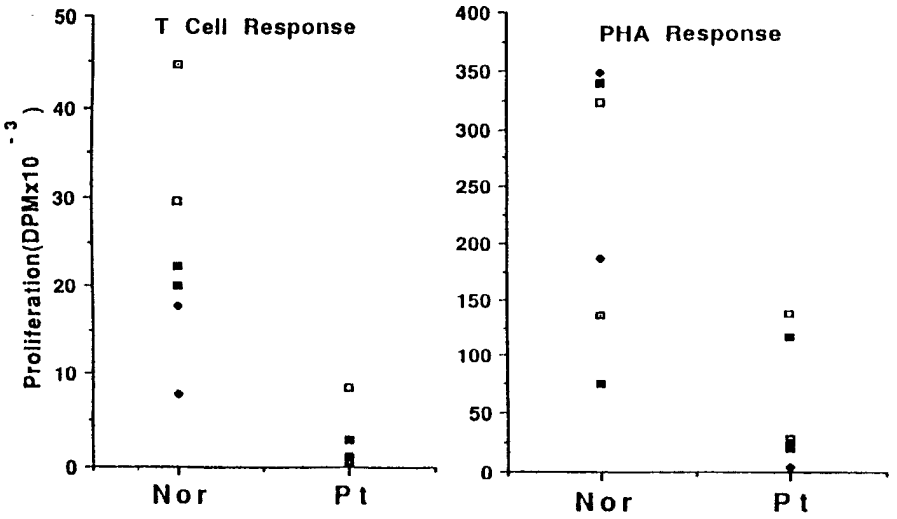

Fig. 2: T cell proliferation in monocyte (MØ)-free system is also depressed ($p < 0.027$) in a group of trauma patients having depressed ($p < 0.027$) phytohemagglutinin (PHA) responses. Normals (Nor) and patients (Pt) peripheral blood mononuclear cells (PBMC) (PHA response) and MØ-depleted E rosetted T cells (T cell response) ($2x10^5$ cells/200 µl/well) were assessed for proliferation (^{3}H-thymidine incorporation) in 72 h of culture in response to PHA and immobilized anti-CD3 (1.5 µg/well) + anti-CD4 (1 µg/well), respectively, and expressed as DPM

proliferation and a depressed level of T cell proliferation at different post-injury days (Fig. 3). These data imply that, although MØ dysfunction may be solely mediating some post-trauma depression of induction of T cell proliferation, a true T cell defect may also be present in some severely injured patients. Whether this defect is a result of increased T cell production of inhibitors such as TGFß or a T cell deficit is unresolved.

In those trauma patients with depressed mitogen-induced proliferation but normal T cell proliferation responses, a variety of MØ aberrations could be responsible for the depression of the mitogen response in the whole PBMC population. The MØ could have decreased levels of costimulatory molecules or cytokines which decrease the levels of mitogen induced activation [22, 19]. In addition, post-trauma MØ have already been shown to have increased population of inhibitors such as PGE_2 and TGFß_ which can inhibit T cell proliferation both indirectly, by reducing MØ costimulatory ligand expression, and directly, by down-regulating T cell IL-2 production and IL-2R expression [24-26].

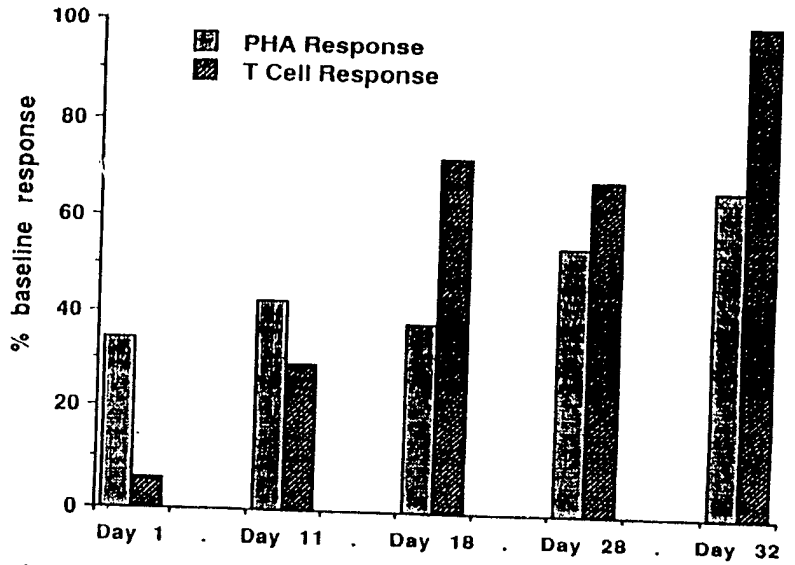

Fig. 3: Proliferation of peripheral blood mononuclear cells (PBMC) phytohemagglutinin (PHA, response) and monocyte (MØ)-depleted E rosetted T cells (T cell response) (2×10^5 cells/200 µl/well) from one patient at different post-injury days in response to PHA and anti-CD3 (1.5 µg/well) + anti-CD4 (1 µg/well), respectively, as assessed by ^{3}H-thymidine incorporation and the % baseline response was calculated as 100 - (A-B)/A x 100, where A = proliferation of paired normal and B = proliferation of the patient

Examination of Interleukin-10 Production During Mitogen-Induced Proliferation

Another MØ inhibitor which could be depressing T cell proliferation is the newly described cytokine IL-10. IL-10 is produced by MØ, T cells, and B cells [10, 11]. Human $CD4^+$ Th2 lymphocytes produce the majority of T cell-derived IL-10 but $CD8^+$ and $CD4^+$ Th1 lymphocytes can also produce IL-10 under certain induction regimens [10-12]. During the PHA-induced proliferation of T lymphocytes in a PBMC population, MØ are the primary producers of IL-10 [30]. Since post-trauma MØ had already been identified as overproducing other inhibitors of T cell proliferation, it seemed likely that post-trauma MØ IL-10 production might also be increased and contribute to depression of post-trauma T cell proliferation. A report of increased IL-10 production by murine splenocytes in a hemorrhage model had also appeared [17]. The levels of IL-10 in supernates of cultures of PBMC from trauma patients with depressed PHA-induced proliferation was assessed with an IL-10 ELISA. Simultaneously, MØ were isolated, and their supernate IL-10 levels, both unstimulated and in vitro stimulated with the gram-positive bacterial cell wall analogue, muramyl dipeptide (MDP), were assessed. In marked contrast to our expectations, the isolated post-trauma MØ IL-10

Table 2: Depressed interleukin-10 production by trauma patients' monocytes
Monocyte IL-10 (ng/10^6 monocytes/ml)

	Normal (n = 37)		Patient (n = 37)	
	Unstimulated	MDP + medium	Unstimulated	MDP + medium
Median	0.910	2.553	0.128	0.461
Range	(0.140-3.781)	(0.715-10.466)	(0-1.325)	(0-2.284)
P			0.0001	0.0001

IL-10 levels in the monocyte culture supernates were measured by ELISA; a total of 37 samples were collected from 24 trauma patients at different post-injury days; monocytes ($3x10^6$ cells/3 ml) cultured in medium alone for 16 h; monocytes ($3x10^6$ cells/3 ml) cultured in medium + MDP (20 μg/ml) for 16 h; statistical significance (p) between normal and patient values determined by Wilcoxon non-parametric test.

levels, both induced and uninduced, were significantly depressed as compared to paired normals' MØ (Table 2). IL-10 levels in PHA stimulated patient PBMC populations were also depressed concomitant to depressed T cell proliferation (Table 3). These data indicated that increased MØ IL-10 could not be the mediator of the depressed PHA responses seen in these patients. At this conference a report of increased IL-10 levels in septic patients' plasma was presented [31]. Since these are pooled patient data, it is unclear whether this increased IL-10 represents an appropriate response to septic challenge in patients with normal immune responsiveness, as seen in trauma patients with increased PHA responses, or whether some other IL-10 cell source besides the PBMC population is responsible. Our data shows that individual trauma patients' PBMC with depressed mitogen responses have depressed, not increased, IL-10 levels in the PBMC population. These patients' depressed mitogen responses cannot, therefore, be attributed to increased IL-10 levels. In addition, the many reports of elevated tumor necrosis factor α (TNFα), IL-8, and IL-6 levels in the plasma of both septic and trauma patients would seem to be in conflict with the concomitant presence of high IL-10 levels since IL-10 effectively down-regulates TNFα, IL-8, and IL-6 at the mRNA level [10, 13]. It is clear from our data, however, that the depressed mitogen responses seen in post-trauma patients' PBMC are not due to increased MØ or lymphocyte IL-10 production.

Investigation of Interleukin-12 Effects on Patient T Cell Proliferation

The decreased mitogen responses of trauma patients' PBMC might be a function of a failure of patients' MØ to adequately stimulate T lymphocytes, as well as an increase in MØ inhibitor production. A post-trauma decrease in MØ activation of T lymphocytes has been demonstrated in a number of murine systems [6, 8]. IL-12 is a newly described cytokine produced by MØ and B lymphocytes which increases the proliferation and differentiation of Th1 type CD4 lymphocytes [32]. IL-12 has been shown to increase the proliferation of PHA-induced

Table 3: Depressed interleukin-10 production by immunosuppressed trauma patients' peripheral blood mononuclear cells.

Experiment No.	Decrease proliferation (%)	Interleukin-10 (ng/10^6 PBMC/ml)	
		Normal	Patient
1	65	4.005	1.850
2	81	2.850	0.320
3	42	3.600	< 0.005
4	96	2.040	0.460
5	66	4.520	0.380
6	98	4.600	< 0.005
7	85	5.100	0.360
8	77	4.600	0.790
9	56	4.600	1.695
p			0.0022

PBMC, peripheral blood mononuclear cells.
Calculated from the counts of phytohemagglutinin (PHA) blasts experiment using the formula A-B/A x 100 where A = counts for normal, B = counts for patient; and the patients having > 35% decrease in proliferation were considered immunosuppressed.
PBMC were cultured in 96 well plate ($2x10^5$ cells/200 μl/well) in the presence of PHA. After 24 h of culture, supernates were harvested and assessed for IL-10 by ELISA. Nine samples were collected from five patients at different post-injury days. Statistical significance (p) between normal and patient values was determined by Wilcoxon nonparametric test.

$CD4^+$ T lymphocytes [16, 33]. HIV^+ patients who, like trauma patients, are characterized by excessive TNFα production and depressed mitogen responses, have been reported to have depressed MØ production of IL-12 [34, 35]. Addition of IL-12 to immune depressed lymphocytes from HIV infected individuals partially restores their responses [34]. In view of these data, it seemed possible that IL-12 addition might restore trauma patients' depressed mitogen responses similar to the mitogen response restoration reported for IL-2 addition to PBMC from trauma patients [2, 3]. As can be seen in Fig. 4, addition of IL-12 did not restore the depressed PHA-induced proliferation of trauma patients' MØ to levels completely comparable to paired normals. However, there was a partial restoration of the suppressed proliferative response in those patients whose MØ-independent T cell proliferation was normal (Fig. 4). In striking contrast, trauma patients with massively depressed T cell proliferation showed no increase in proliferative capacity with in vitro IL-12 addition (Fig. 5). These data have a number of possible interpretations which need to be further explored. The most straightforward interpretation is that some trauma patients experience a total T cell dysfunction that effects $CD4^+$ Th1, $CD4^+$ Th2 and, consequently, the $CD8^+$ cell proliferation. Since IL-12 is known to augment proliferation in both CD4+ and CD8+ T lymphocytes, the

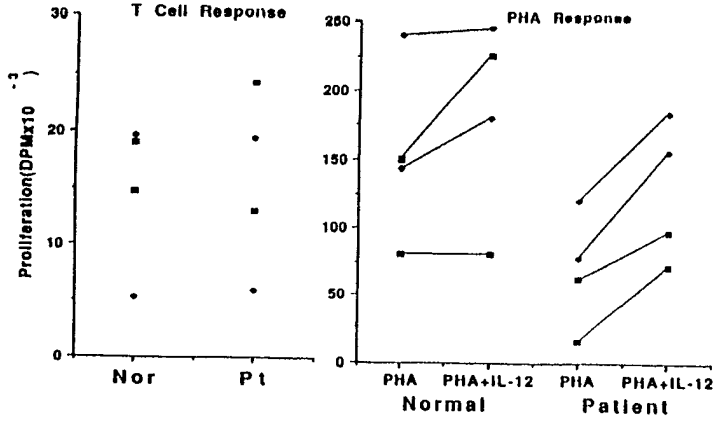

Fig. 4: Partial restoration of phytohemagglutinin (PHA) responses by interleukin-12 (IL-12) in patients who have normal T cell responses in monocyte (MØ)-free system. Normals (Nor) and patients (Pt) PBMC (PHA response) and MØ-depleted E rosetted T cells (T cell response) ($2x10^5$ cells/200 µl/well) were assessed for proliferation (^{3}H-thymidine incorporation) in 72 h of culture in response to PHA and immobilized anti-CD3 (1.5 µg/well) + anti CD4 (1 µg/well), respectively, and expressed as DPM. IL-12 was added in PHA experiments at a concentration of 100 U/ml.

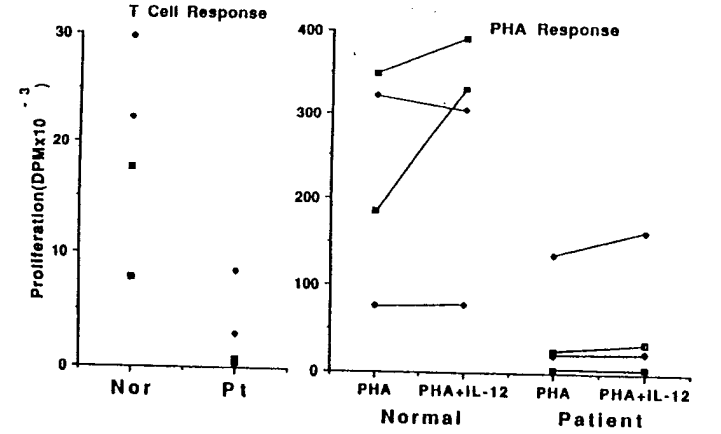

Fig. 5: Interleukin-12 (IL-12) fails to restore phytohemagglutinin (PHA) responses in patients who have depressed ($p < 0.027$) T cell responses in monocyte (MØ)-free system. Normals (Nor) and patients (Pt) PBMC (PHA response) and MØ-depleted E rosetted T cells (T cell response) ($2x10^5$ cells/200 µl/well) were assessed for proliferation (^{3}H-thymidine incorporation) at 72 h of culture in response to PHA and immobilized anti-CD3 (1.5 µg/well) + anti-CD4 (1 µg/well), respectively, and expressed as DPM. IL-12 was added in PHA experiments at a concentration of 100 U/ml.

failure to detect any IL-12 mediated improvement in PHA induced PBMC proliferation may reflect a defect in both these lymphocyte populations [14-16]. Alternatively, the failure of IL-12 to improve this group of patients' PHA response could reflect an overwhelming presence in PBMC of MØ and/or T cell derived inhibitors which cannot be contravened by the positive stimulus provided by IL-12. This excessive inhibitor interpretation seems a less likely explanation for the total lack of T cell stimulation seen when examining this patient groups' anti-CD3-CD4 stimulated proliferation of purified T cells (Fig. 5). Th2 lymphocytes are more strongly stimulated by anti-CD3 than Th1 lymphocytes because they are less dependent on costimulatory signals [11, 22]. In addition, Th2 lymphocyte proliferation is less sensitive to inhibitory mediators such as PGE_2 and TGFß and Th2 cells would see much reduced concentrations of these mediators in the MØ-free system. Those data showing partial IL-12 mediated restoration of the patients' PBMC response to PHA in cases in which the T cell proliferation was essentially normal could, therefore, be interpreted to reflect that patients' Th1 population is more easily affected by MØ inhibitors and lack of MØ costimulation and this Th1 activity can, therefore, be partially restored with IL-12 addition. However, when a true T cell deficiency occurs, it may affect Th2 as well as Th1 lymphocytes. A depletion of T cells in the PBMC population has been suggested as mediating depressed T cell mitogen responses in some post-trauma systems [21, 36]. An important consideration in the PBMC experiments is that there may be different ratios Th1 to Th2 to MØ to B cells. However, in the MØ-independent T lymphocyte system, the purified T lymphocytes have been adjusted so that total numbers are the same between patients and normals. A trauma induced depletion of responding T cells cannot therefore account for decreased proliferation capacity in the T cell system. The ratios of $CD4^+$ Th1 to Th2 to $CD8^+$ lymphocytes may vary in the T cell system, however.

Conclusions

These data suggest that the depressed mitogen-induced proliferation of some trauma patients' PBMC are the result of a number of dysfunctions which can be more or less severe. Post-trauma depressed mitogen response in patients' PBMC result from MØ dysfunction which then can mediate a T lymphocyte proliferative depression. Once the MØ are removed and the T lymphocytes stimulated directly through a costimulator independent system, the T cells can respond normally. Addition of the Th1 stimulator, IL-12, can partially replenish proliferative capacity to the costimulator-dependent PHA response. The costimulator-dependent defect in PBMC proliferation may reflect increased MØ PGE_2 or TGFß production. However, the data clearly show that loss of these patients' PBMC proliferative capacity to PHA is not a result of MØ or T cell overproduction of IL-10. This group of patients has no actual T cell proliferation deficit but only a loss of T cell proliferation in a costimulator-dependent system. In contrast, some patients' post-trauma immune dysfunction proceeds to produce an actual loss of T lymphocyte function which appears to affect both Th1 and Th2 lymphocytes as reflected by a loss of T cell proliferation in a costimulator-independent system. When patients exhibit this extensive T cell defect, IL-12 has no ability to restore the PBMC proliferative response to PHA. It is important to distinguish the two types of immunosuppression which can occur in the post-trauma patient mitogen responses since the resulting immune deficits are quite different. However, both these post-trauma immune dysfunctions manifest themselves in the same loss of PBMC mitogen responsiveness. Additionally, any immunomodulation directed to these two types of immune dysfunctions

must be targeted at different cell populations. Continued assessment of patients' immune status needs to be made since one patient may experience a loss of T cell costimulator-dependent proliferation and then progress to the more severe loss of total T cell proliferation. It remains to be seen if therapeutic intervention can slow or reverse this progression or whether each immune deficit needs to be treated as an independent occurrence.

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